Institut für Veterinärphysiologie

der Vetsuisse–Fakultät Universität Zürich

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The Role of Calcitonin Gene-Related Peptide in the Endothelin-1 mediated Pain Perception

Inaugural-Dissertation

zur Erlangung der Doktorwürde der

Vetsuisse-Fakultät Universität Zürich

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Zürich 2013

CONTENTS

SUMMARY

In vessels calcitonin gene-related peptide (CGRP) has an anti-endothelinergic effect by terminating the binding between the endothelin A (ET-A) receptor and endothelin 1 (ET-1). As ET-1 can elicit pain through ET-A receptors on nociceptors, we tested the hypothesis that CGRP might similar as in vessels promote dissociation of the ET-1/ET-A receptor complex on nociceptors. This way CGRP could have analgesic effects in the periphery.

Calcitonin receptor-like receptor transgenic mice (CLR-tg) showed a significant decreased sympathetic response during thermal stimulation in etomidate anaesthesia. Baseline values of behaviourally assessed mechanical and thermal sensitivity were also decreased, but no difference was observed after induction of hyperalgesia compared to the control mice. αCGRP-knockout ($\alpha CGRP^{-/-}$) mice showed an increased nociception during thermal stimulation in etomidate anaesthesia, but no difference compared to their wild-type controls was observed for behaviourally assessed mechanical and thermal sensitivity neither during baseline conditions nor after induction of hyperalgesia.

Intraplantar ET-1 injection confirmed that $\alpha CGRP^{-/-}$ mice are more sensitive to this pain-inducing peptide, whereas the CLR-tg mice have a decreased nociception compared to their respective controls.

Our findings suggest that CGRP may have an analgesic effect on the peripheral endings of the nociceptors by modulating the interaction between ET-1 and ET-A receptors, as described in vessels.

ZUSAMMENFASSUNG

In Blutgefässen hat calcitonin gene-related peptide (CGRP) eine anti-endothelinergische Wirkung, in dem es die Bindung zwischen Endothelin-A (ET-A) Rezeptor und Endothelin-1 (ET-1) beendet. Da die Aktivierung von ET-A Rezeptoren auf Nozizeptoren Schmerz verursacht, haben wir die Hypothese getestet, dass CGRP, wie in den Blutgefässen, die Dissoziation vom ET-1/ET-A Rezeptor Komplex fördern kann. CGRP hätte damit eine analgetische Wirkung in der Peripherie.

In Etomidat Anästhesie zeigten die Calcitonin receptor-like receptor transgene (CLR-tg) Mäuse eine reduzierte sympathische Antwort auf Hitze-Stimulation. Verhaltensbasierte Basismessungen von mechanischer und Wärmeempfindlichkeit waren auch reduziert, aber es konnte kein Unterschied nach Induktion von Hyperalgesie gegenüber den Kontrollmäusen festgestellt werden. αCGRP-knockout ($\alpha CGRP^{-/-}$) Mäuse zeigten eine erhöhte Nozizeption bei der Hitze-Stimulation in Etomidat Anästhesie. Wir konnten aber keinen Unterschied feststellen, weder in den Basismessungen von mechanischer und Wärmeempfindlichkeit, noch nach induzierter Hyperalgesie .

Die intraplantare Injektion von ET-1 bestätigte eine erhöhte Schmerzempfindlichkeit der $\alpha CGRP^{-/-}$ Mäuse und eine reduzierte Schmerzempfindlichkeit der CLR-tg Mäuse, im Vergleich zu den entsprechenden Kontrollmäusen.

Unsere Ergebnisse deuten darauf hin, dass CGRP eine analgesische Wirkung an den peripheren Enden der Nozizeptoren verübt, in dem es die Interaktion zwischen ET-1 und ET-A Rezeptor moduliert.

1. INTRODUCTION

1.1. Pain

1.1.1. Definition

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage".

1.1.2. Function of pain sensation

The capacity to avoid acute painful stimuli is essential to survive and for wellbeing. Protective reflexes occuring during an injury minimize potential tissue damage and hence prolong survival. An individual that can not feel pain, for example after different kind of injury, will not rest the lesion and consequently acquire serious health problems. Individuals with a congenital absence of the sense of pain are very rare. An example is the "channelopathy-associated insensitivity of pain" where the loss of function of the voltage-gated sodium channel gene SCN9A lets the individuals perceive the sensations of touch, warm and cold temperature, proprioception, tickle and pressure, but not painful stimuli (Cox et al., 2006). SCN9A encodes $Na_v1.7$, the alpha subunit of a tetrodoxin-sensitive voltage-gated sodium channel, expressed mostly in peripheral sensory neurons, where they are involved in the propagation of action potentials (Cox et al., 2006). Preterm mortality of these individuals is not described, but the constantly hazardous situations in which these individuals live, because of their incapability to learn avoid risky behaviour, demonstrate how important it is to have an intact perception of pain.

1.1.3. Classification of pain

Different types of pain classification exist in literature, depending for example on the type of origin (Cervero & Laird, 1991; Schaible & Richter, 2004), on the type of nociceptive stimulus (chemical, thermal or mechanical), on a time-factor (acute versus

chronic pain), or on the localization of pain (somatic superficial pain, somatic deep pain, visceral pain; McMahon & Koltzenburg, 2006).

The classification of Cervero and Laird seems to be the most complete in term of description the different aspects of pain perception (Cervero & Laird, 1991). They distinguish three different pain types:

1. The acute physiological nociceptive pain occurs when a noxious stimulus is applied to normal tissue. It is also called "normal" pain, because it is part of the healthy mammalian sensory repertoire. This kind of pain is a prerequisite to survive and preserve an undamaged body, because normally withdrawal reflexes are induced.

2. The pathophysiological nociceptive pain parallels tissue damage and inflammation and has various manifestations: spontaneous pain (pain sensation without any stimulus[1]), hyperalgesia (increased pain from a stimulus that normally provokes pain[1]) or allodynia (pain due to a stimulus that does not normally provoke pain[1]). Pathologic pain can also be subclassified into inflammatory pain or neuropathic pain, depending on the cause (Lamont et al., 2000).

3. The neuropathic pain is caused by injury or disease of the neurons in the central and / or peripheral nervous system (Cervero & Laird, 1991, Schaible & Richter, 2004). Many diseases can cause neuropathic pain, e.g. diabetes mellitus, herpes zoster, or the complex regional pain syndrome (CRPS). The latter involves a dysregulation of the autonomic nervous system, where CNS representations of the sympathetic nervous system, the somatosensory system, and the somatomotor system are altered (Jänig & Baron, 2003).

A definitive classification of pain is not yet fully established. The division of pain in three different types is simplified, because often one component does not exclude the other, e.g. inflammatory and neuropathic factors may be involved simultaneously (Schaible, 2007).

[1] Definition by IASP

1.1.4. Acute and chronic pain

The distinction between acute and chronic pain characterizes the pain on a temporal level. Acute pain is the first pain, usually sharp in quality and well localized, which begins immediately after injury (McMahon & Koltzenburg, 2006); on the other hand, the definition of chronic pain is not yet established. It was first defined as a pain sensation that persists longer than six months (Russo & Brose, 1998), but this arbitrary time limit is today replaced by specific characteristics of chronic pain. Chronic pain "persists beyond the expected course of an acute disease process" (Russo & Brose, 1998), where the cause of the pain sensation, e.g. tissue damage, is often not identifiable anymore. Chronic pain might be the result of a chronic disease and of persistent nociceptive processes. Its relevance is not only about the pain sensation per se but also the involvement of affective and cognitive components and their interplay with social factors, which promote a situation of destructive stress, characterized by neuroendocrine dysregulation, fatigue, dysphoria and impaired physical and mental performances (Schaible & Richter, 2004; Chapman & Gavrin, 1999).

1.1.5. Nociception and nociceptors

In line with Schaible and Richter the pain system should be better called the nociceptive system, because pain is the subjective, conscious experience of nociception (Schaible & Richter, 2004). In contrast, nociception is merely the neural process of encoding and processing of a noxious stimulus (definition by IASP). Nociception and pain can occur without each other. During surgery, for example, there is an activation of the nociception system, but pain is absent, because the patient is anesthetized (local or general anesthesia).

The idea that special peripheral nerve afferents respond to mechanical, thermal or chemical stimuli, conducting pain sensation, was first postulated by Sherrington in 1906 (Sherrington, 1906). These afferents were then called nociceptors.

Nociceptors are pseudo-unipolar neurons; their cell body are located in the dorsal root ganglia or in the trigeminal ganglia, and they have both central and peripheral processes. Their peripheral axons ramify into the tissue until the skin, where they

transduce nociceptive stimuli to the spinal cord dorsal horn through their central processes. Stimulation of nociceptors happens only if the stimulus intensity reaches the noxious range (McMahon & Koltzenburg, 2006).
As for the classification of pain, nociceptors can be classified according to different criteria (McMahon & Koltzenburg, 2006):

1. Cytological feature: myelinated and unmyelinated fibers
2. Modality of stimulation that evoke response: thermal, mechanical, chemical
3. Response characteristics
4. Distinctive chemicals markers

The most common classification of nociceptors is the distinction between myelinated and unmyelinated fibers. The two main nociceptors in these classes are the unmyelinated C-fibers and the thinly myelinated Aδ fibers.
C-fibers are polymodal, therefore they respond to a variety of different stimuli such as thermal or mechanical ones. They do not have any myelin sheath and their conduction velocity is about 2 m/sec. Normally the pain elicited by these fibers is poorly localized and is often called “second” or “slow” pain. A subclass of C-fibers are the peptidergic C-fibers, which are able to produce and release neuropeptides (such as calcitonin gene-related peptide (CGRP), substance P (SP), or neurokinin A) after noxious stimulation, causing neurogenic inflammation.
Myelinated Aδ-fibers have a conduction velocity of 6-30 m/sec and mediate the acute, well-localized “first” or “fast” pain. They are subdivided in type I and type II, where type I fibers respond to mechanical and chemical stimuli and have a high threshold for thermal pain. In contrast, type II fibers have a lower heat threshold but a very high mechanical threshold (Lamont et al., 2000; McMahon & Koltzenburg, 2006; Basbaum et al., 2009).

Regarding the nociception, there is a sequence of events distinctive for this process (Kuner, 2010; Schaible & Richter, 2004; *see Fig. 1*)

1. A noxious stimulus activates nociceptors (Aδ- or C-fibers) in the peripheral tissue. The nociceptor traduces the mechanical, chemical or thermal stimulus

into membrane depolarization. The latter is amplified by sodium channels and an action potential is evoked (Kuner, 2010).

2. The action potential is conducted to the dorsal horn or brainstem.
3. Dorsal horn neurons, interneurons or ascending tract neurons, are activated by the nociceptors.
4. Ascending axons in the spinothalamic tract activate the thalamocortical system, which produce the conscious sensation of pain.

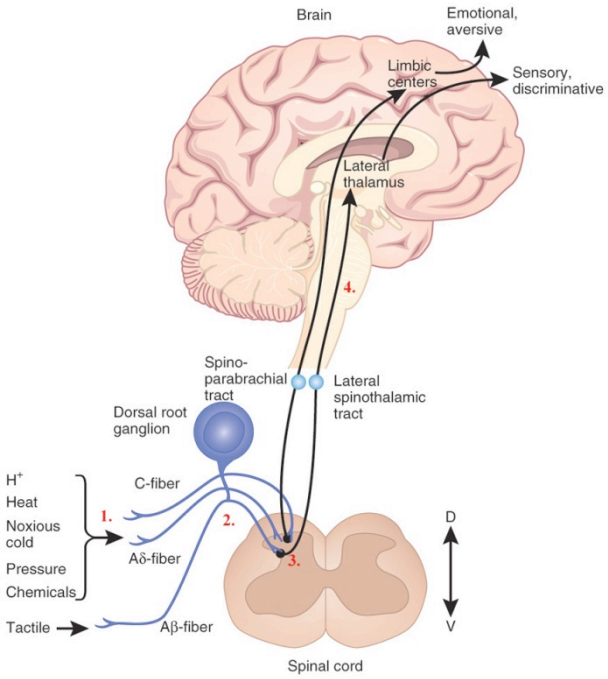


***Figure 1**: General nociceptive pathway (modified from Kuner, 2010)*

1.2. Calcitonin gene-related peptide (CGRP)

1.2.1. Discovery and distribution

Calcitonin gene-related peptide (CGRP, later named αCGRP) is a 37-amino acid neuropeptide, first discovered in 1983 in rats (Rosenfeld et al., 1983). CGRP is generated by the gene encoding for calcitonin (CT) by tissue-specific alternative mRNA splicing (Rosenfeld et al., 1983; Rosenfeld et al., 1984; Amara et al., 1982; Amara et al., 1984). Rat and human CT have a 23% homology in the amino acid sequence with the rat αCGRP, whereas the salmon CT has a 30% homology in the amino acid sequences with rat αCGRP (Bell & McDermott, 1996). The production of CT mRNA is limited to the C-cells of the thyroid gland, while αCGRP mRNA is yielded in different cell types of the central and the peripheral nervous system (Amara et al., 1982; Rosenfeld et al., 1983).

In addition to αCGRP, a second CGRP form was subsequently identified and called βCGRP (Amara et al., 1985; Hoppener et al., 1985). Both belong to the calcitonin family, together with other three known peptides: calcitonin, amylin and adrenomedullin (Poyner et al., 2002). In rats, αCGRP and βCGRP differ by one amino acid (a lysine for glutamic acid in position 35) and present a high sequence homology, but they do not stem from the same origin gene (Amara et al., 1985; Wimalawansa, 1996). In mammals, αCGRP is more abundant than βCGRP (Brain & Grant, 2004).

CGRP is widely distributed in the peripheral and central nervous system (Brain & Grant, 2004). Nerves that produce and store CGRP are able to release the neuropeptide from the peripheral as well as the central axons. Immunoreactivity to CGRP is mostly seen in myelinated Aδ fibers and small unmyelinated sensory C-fibers. In the latter, CGRP is often co-stored with substance P and neurokinin A (Lundberg et al., 1985), and the release of substance P is potentiated by CGRP in the rat spinal dorsal horn during mechanical nociception (Oku et al., 1987). A lower amount of CGRP is co-localized with acetylcholine in the motor neurons (Ma, 2004; Bell & McDermott, 1996). In contrast to αCGRP, βCGRP is localized almost exclusively in the enteric nervous system and in the pituitary gland (Brain & Grant,

2004; Petermann, 1987; Mulderry et al., 1988), where it regulates, among others, the gastric acid secretion and blood flow (for review see Van Rossum et al., 1997).

The fact that CGRP plays a very important role in the regulation of the vascular tone (see *1.2.3 Functions of CGRP in the cardiovascular system*) is evident from the perivascular localization of nerve fibers producing and storing CGRP. These nerves do not have solely an afferent function, transmitting the peripheral stimuli to the central nervous system, but also an efferent vasodilator effect (Bell & McDermott, 1996). In fact, the endings of the nerves innervating small arteries penetrate into the vascular smooth muscle layer and release CGRP that exerts local effects (Holzer, 1992). In the heart, CGRP-containing nerve fibers are more abundant in the atria - especially in the right atrium - than in the ventricles (Wimalawansa, 2001), which suggests together with other additional locations of CGRP expression that this neuropeptide plays multiple physiological roles in the organism.

1.2.2. Calcitonin gene-related peptide receptor

The CGRP receptor consists of: i) a G-protein-coupled receptor, the calcitonin receptor-like receptor (CLR), ii) a receptor-activity-modifying protein (RAMPs), and iii) a receptor component protein (RCP), essential for correct biological function (Arulmani et al., 2004; *see Fig. 2*). The co-expression of CLR with a specific RAMP variant determines the ligand specificity and the function (McLatchie et al., 1998). Three different RAMPs are known: RAMP1, RAMP2 and RAMP3. Association of CLR with RAMP1 results in a functional and specific receptor for CGRP, the protein-complex of CLR with RAMP3 recognizes both CGRP and adrenomedullin, and the complex CLR/RAMP2 binds preferentially adrenomedullin (Hay et al., 2008).

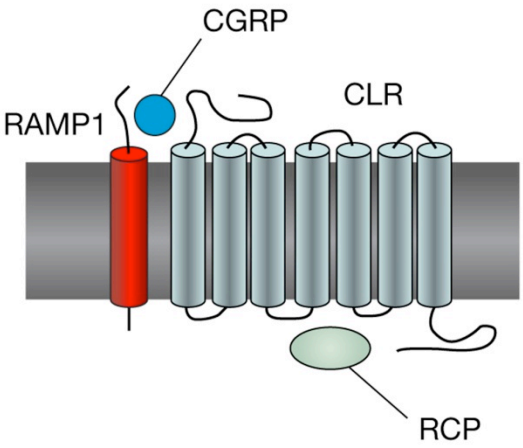


***Figure 2**: Schematic illustration of the CGRP receptor. Calcitonin gene-related peptide: CGRP; receptor-activity-modifying protein 1: RAMP1; calcitonin receptor-like receptor: CLR; receptor component protein: RCP (modified from Raddant & Russo, 2011)*

Similar as to CGRP its receptor is also widely distributed throughout the nervous system but also the cardiovascular, gastrointestinal, respiratory, endocrine, musculoskeletal, and trigeminal system (for review see Arulmani et al., 2004; Villalón & Olesen, 2009; Hagner et al., 2002).

1.2.3. Functions of CGRP in the cardiovascular system

CGRP is one of the most potent vasodilators (Brain & Grant, 2004). In the cardiovascular system, CGRP acts via endothelium-dependent and -independent mechanisms. In the first one, activation of the CGRP receptor promotes the increase of the cAMP level. In turn, cAMP promotes vasodilatation via PKA, which stimulates the NO synthase, that leads to the release of nitric oxide (NO) (Brain & Grant, 2004). NO acts on smooth muscle cells and causes relaxation (Wimalawansa, 1996; Villalón & Olesen, 2009). In the endothelium-independent mechanism, where endothelium is absent, CGRP released by the perivascular nerve endings acts directly on the smooth muscle cells, increasing the cAMP level through the activation of the adenylate cyclase (Hirata et al., 1988). As a consequence, intravenously administered CGRP causes a decrease of the mean arterial blood pressure and an increase of the heart rate (Gennari & Fischer, 1985).

Apart from that CGRP has also other effects on the heart and on the cardiovascular system. CGRP is a positive chronotropic and inotropic factor, it increases the coronary blood flow permeability, has cardioprotective effects through preconditioning, and regulates angiogenesis (Opgaard et al., 2000; Li & Peng, 2002; Wimalawansa, 2001).

1.2.4. Functions of CGRP in the nervous system

The localization of CGRP in small dorsal root ganglion neurons and in nociceptive fibers suggests that CGRP plays an important role in modulating the pain perception (Powell et al., 2000; Van Rossum et al., 1997), in potentiating the excitatory actions (enhancing the release of SP), and as neurotrophic factor, increasing the synthesis of acetylcholine receptor (Rossi et al., 2003). Indeed, after noxious thermal and mechanical stimulation, CGRP is released centrally in the superficial dorsal horn (Morton & Hutchison, 1989) and peripherally in the skin (Kilo et al., 1997; Kress et al., 1999). The CGRP release from the nociceptors in the periphery is stimulated by depolarization of the nociceptor or by the activation of the capsaicin receptor Transient Receptor Potential Vanniloid 1 (TRPV1). TRPV1 is an ion channel that belongs to the superfamily of Transient Receptor Potential. One function of these receptors on the nociceptors is to translate physical and chemical noxious stimuli in action potentials (Kessler et al., 1999; Kichko & Reeh, 2009). In turn, CGRP causes vasodilatation in the skin but does not increase the permeability of the microvasculature (Poyner, 1992). The latter is mediated by SP and neurokinin A (Holzer, 1998). Furthermore, the molecular action of CGRP in the nervous system includes closing potassium channels, resulting in a prolonged increase in the excitability of the cells (Poyner, 1992).

In terms of neurogenic inflammation, the involvement of the immune system is another function of CGRP in addition to the vasodilatation. Indeed, together with SP, it stimulates the adhesion of leukocytes to the vessel wall and their emigration to the inflamed tissue (Walsh et al., 1995; Holzer, 1998).

To understand the complexity of the physiological role of CGRP, not only cellular mechanisms and molecular interplays are important research subjects but also effects on the whole organism. Therefore different studies were carried out to investigate

behavioural responses in rodents after injection of CGRP and its antagonist (CGRP8-37) into different body compartments.

Yu et al. summarize in their review (Yu et al., 2009) the different action of CGRP and CGRP8-37 in the dorsal horn and in the brain. Contradictory findings about the pro-algetic role of CGRP in the spinal cord are still discussed in the literature. Oku et al. demonstrated that intrathecally injected CGRP causes mechanical hyperalgesia in the spinal cord (Oku et al., 1987), but in other two studies this effect was not observed (Yu et al., 1994; Jolicoeur et al., 1992). In contrast, the antinociceptive effect of CGRP8-37 in the spinal cord is widely accepted (Yu et al., 1996a; Yu et al., 1996b; Salmon et al., 2001). In the brain, administration of CGRP to specific brain structures involved in pain processing (i.e. periaqueductal grey, nucleus raphe magnus, central nucleus of amygdala, or nucleus accumbens) resulted in an antinociceptive effect (Yu et al., 2009).

In addition, to better understand the role of CGRP in the pain perception, a CT/αCGRP knockout mouse was produced and tested in the nociceptive responses. The CT/αCGRP$^{-/-}$ mice showed normal pain responses to thermal stimulation (hot plate test and radial heat) in the baseline measurements. After kaolin/carrageenan induced arthritis of the knee joint, however, the expected secondary hyperalgesia did not develop like in αCGRP$^{+/+}$ control mice and CT/αCGRP$^{-/-}$ mice did not show a decreased paw withdrawal threshold. This indicates that CGRP may be involved in spinal and peripheral pain pathways during inflammation (Zhang et al., 2001), like a neuromodulator, but how exactly this modulation works remains unclear.

All these studies indicate that the role of CGRP in the pain system is very complex and not completely understood. Behavioural data and neurochemical studies strongly suggest that CGRP in the spinal cord promotes nocifensive behaviours, but further investigations are required to gain a clear and complete knowledge about the function of CGRP in the pain system, focused above all on the possible development of analgetic drugs.

1.2.5. Role of CGRP in migraine pathophysiology

Numerous studies have been carried out with the purpose to understand the role of CGRP in the pain mechanism. After the discovery of the neuropeptide in 1983 by Rosenfeld et al., other studies were performed to describe its localization in the body. Regarding migraine pathophysiology an important discovery was that CGRP is highly abundant in trigeminal nerve bodies and perivascular nerve fibers of the cerebral circulation (Uddman et al., 1985). The role of CGRP in migraine was further confirmed when elevated CGRP levels, but not of other peptides, were found in blood plasma during migraine attacks (Goadsby et al., 1990; Juhasz et al., 2003). Consequently, CGRP is now regarded as key player in migraine pathology, although more recent publications contradict this latter study, where no significant increase of CGRP plasma levels were measured during headache (Tfelt-Hansen & Le, 2009; Tvedskov et al., 2005). However, two other points confirm the hypothesis that CGRP plays a key role in migraine: i) the intravenous injection of CGRP in migraine patients causes headache symptoms (Lassen et al., 2002), and ii) CGRP-receptor antagonists (olcegepant and telcagepant) have been proven to be effective against acute migraine attacks (Olesen et al. 2004; Ho et al., 2008a; Ho et al. 2008b). The dilatation of the trigeminovascular vessels after CGRP release seems to be the leading cause of pain sensation, but which stimulus induces the release of CGRP remains unknown (Villalón & Olesen, 2009).

(For reviews concerning all the functions of CGRP in mammalian body see Brain & Grant, 2004; Arulmani et al., 2004; Bell & McDermott, 1996)

1.3. Endothelin (ET)

1.3.1. Discovery

The family of endothelins consists of three known 21-amino acid long peptides: endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3), that are encoded by three different genes (Khodorova et al., 2009a). Most widely expressed endothelin is ET-1 and its distribution is overlapping with ET-2 and ET-3 (Sokolovsky, 1991; Khodorova et al., 2009a).

ET-1 was first discovered as a product of endothelial cells and to have strong vasoconstrictive effects (Yanagisawa et al., 1988). Later on, other cell types were recognised to have the ability to synthesize and release ET-1; for example epithelial cells and keratinocytes after tissue injury (Ahn et al., 1988; Yohn et al., 1993), cardiomyocytes (Ito et al., 1993), macrophages (Ehrenreich et al., 1990) or leucocytes (Sessa et al., 1991). Interestingly, ET-1 is also oversecreted by cancer cells such as metastatic prostate cancer cells and might even specifically mediate cancer pain (Nelson et al., 1995).

1.3.2. ET-1 receptors

Two G-coupled receptors mediate the different cellular effects of ET-1: the ET-A receptor (Arai et al., 1990) and the ET-B receptor (Sakurai et al., 1990). ET-B receptors seem to have the same affinity for all three endothelins, whereas the ET-A receptor interacts preferentially with ET-1 and ET-2 rather than ET-3 (Pomonis et al., 2001).

1.3.3. ET-1 function in the cardiovascular system

ET-A and ET-B receptors are G-coupled receptors and have both opposite and overlapping functions (Khodorova et al., 2009a). In the cardiovascular system, the ET-A receptor is present throughout the vascular smooth muscle cells and mediates a long lasting constriction of the vessels by increasing the intracellular calcium

concentration (Maxwell et al., 1998). In addition, promotion of cell growth, proliferation and migration, and production of reactive oxygen species during inflammation are also mediated by ET-A receptor activation (Meens et al., 2009; Hynynen & Khalil, 2006; Schneider et al., 2007; Kawamata et al., 2008).

In contrast, activated ET-B receptor in endothelial cells causes the release of NO and, therefore, the relaxation of blood vessels (Tirapelli et al., 2005). The functions of these receptors are not restricted to one cell type: the ET-A receptor is also found in endothelial cells, where it mediates the increase of intracellular calcium and, hence, contraction; the ET-B receptor is also present in stomach smooth muscle cells, where it induces contraction (Khodorova et al., 2009a). These few examples underline that ET-1 receptors subtypes do not have a singular function in the cardiovascular system, but can have even opposite effects, depending on the cell type.

1.3.4. Analgesic and pro-algesic functions of ET-1

In addition to the strong presence of ET-1 receptors in smooth muscle cells and endothelial cells, ET-A and ET-B receptors have a broad distribution in the peripheral and central nervous system too (Rubanyi & Polokoff, 1994). Relevant for nociception, ET-A receptors are mostly present in small diameter sensory neurons, C- and Aδ-fibers, whereas ET-B receptors are mainly expressed in dorsal root ganglia-satellite cells, in ensheating Schwann-cells, and also in keratinocytes (Pomonis et al., 2001). The anatomical distribution of ET-1 receptors in cells crucially responsible for pain sensation involves ET-1 in the pain axis. In fact, intraplantar, intraarticular, or subcutaneous injection of ET-1 in different species induces pain sensation, decreases paw withdrawal threshold for mechanical stimulation, and increases licking/biting behaviours in rodents (De-Melo et al., 1998; Hans et al., 2007; Raffa et al., 1996; Khodorova et al., 2009a). The pro-algesic function of ET-1 is due above all to the interaction between ET-1 and ET-A receptors (*see Fig. 3)*, whose activation seem not only to result in the known pro-algesic function, but also induce release of CGRP and glutamate from peripheral nerve endings and keratinocytes (Khodorova et al., 2009a; Piovezan et al., 2000).

Regarding the activation of ET-1 receptors after ET-1 intraplantar injection in rats, Khodorova et al. described two distinct roles of ET-A and ET-B. The activation of ET-A receptor has a proalgesic effect (as mentioned above), while the activation of ET-B receptor is associated with an indirect analgesic function via induction of β-endorphin release from keratinocytes (Khodorova et al., 2002). It is not possible that ET-B receptor has a direct inhibitory effect because this receptor is not present on sensory fibers (Pomonis et al., 2001), which was confirmed later (Khodorova et al., 2003). ET-B receptor activation in keratinocytes induces β-endorphin release in keratinocytes, which binds to the μ-opiate receptor situated on nociceptors and leads to the opening of G-protein-sensitive K^+-channels, lowering the excitability and generation of action potentials (Khodorova et al., 2003).

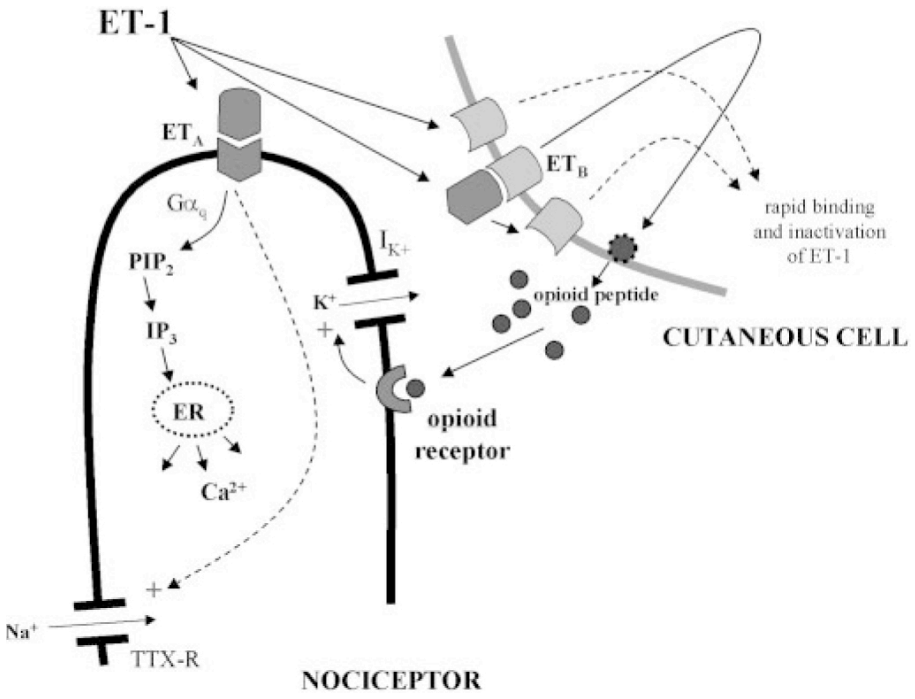


***Figure 3**: Pain specific cellular mechanism of ET-A and ET-B receptor activation. The ET-A receptor activation on nociceptor promotes the increase of intracellular calcium and the activation of the Tetrodoxin-Resistant sodium channel (TTX-R), leading to an increase of intracellular sodium (direct depolarization / increased excitability). On the other hand, the activation of ET-B receptor on keratinocytes induces the release of β-endorphin, which cause an indirect analgesic function through μ-opiate receptors on nociceptors and opening of potassium channels (modified from Khodorova et al., 2002)*

Later, the same research group questioned that ET-B receptor activation solely triggers an analgesic cascade (Khodorova et al., 2009b). Previous studies already indicated a pro-algesic role of ET-B receptor activation. For example, intraperitoneal injection of ET-B receptor agonist in rats results in abdominal constrictions (Raffa et al., 1996) and administration of BQ-788 (ET-B receptor antagonist) suppresses the mechanical hyperalgesia in sensitized mice (Baamonde et al., 2004). Further examples are listed in (Khodorova et al., 2009a) and confirm the involvement of ET-B receptor in the pain pathway.

Khodorova et al. (Khodorova et al., 2009b) pointed out that only the combination of both ET-A and ET-B receptor antagonists were able to completely abolish the mechanical hyperalgesia caused by intraplantar injection of ET-1 in rats. Indeed, the exclusive administration of either ET-A or ET-B receptor antagonist prevented only partially the mechanical hyperalgesia. To explain the apparent contradictory and dual role of ET-B receptors they suggest that the ET-B receptor might have a pro-algesic role when ET-1 is present at low concentration but an anti-hyperalgesic function at high ET-1 concentration (i.e. tissue/keratinocytes damage). Perhaps the receptor distribution on different cell types causes the opposing effects on pain behaviour.

Since ET-A receptor antagonist reduces pain in cancer patients affected from metastatic prostate cancer (Carducci et al., 2002), the study of the ET-1 interaction with the ET-A and ET-B receptors is a promising research field to develop new analgesic drugs. However, the complexity of dual or more effects of the receptors activation suggests that many different factors, like cell and tissue specificity, concentration of ET-1 and condition of the extracellular environment (e.g. inflammation), contribute to the final result. The opposing effects of activated ET-A and ET-B receptors show that further studies are necessary to better understand the whole mechanism of ET-1 mediated pain.

1.4. Aim of the study and hypothesis

1.4.1. Approach

Our hypothesis arose from the observation that after ranking fights, calcitonin receptor-like receptor transgene (CLR-tg) male mice showed much more injuries compared to their wild-type littermates. More detailed monitoring of the mice' daily behaviour confirmed a quite passive behaviour of the CLR-tg mice during conflicts, suggesting a decreased nociception of this genotype. The hypothesis that an overexpression of CGRP receptors on nociceptors influences the pain perception in an analgetic way is a novel approach in the study of pain because up to now, the role of CGRP and his receptor in the central nervous system was believed to be pro-algesic, whereas in the periphery CGRP was only associated as the causing agent of neurogenic inflammation (*see 1.2.4 Functions of CGRP in the nervous system*).

1.4.2. Hypothesis

Recent publications (Meens et al., 2010; De Mey et al., 2009; Meens et al., 2009) confirmed the presence of ET-A and ET-B receptor on smooth muscle cells, on periarterial sensory motor nerves and on the endothelium. The group demonstrated that endogenous CGRP released by periarterial sensory motor neurons (as a result of TRPV1 stimulation) or exogenous administered CGRP, is able to terminate (cAMP independently) the contraction of rat mesenteric arteries induced by ET-1. The long lasting contraction caused by ET-1 in vitro can be prevented by selective ET-A receptor antagonists or mixed ET-antagonists, but a complete reversion of the contraction caused by an already established ET-1/ET-A complex is not possible with ET-A antagonists (Meens et al., 2010). Furthermore, the ET-1-induced vasoconstriction can not be terminated even by washing away the agonist for several times (De Mey et al., 2009). The molecular mechanism whereby CGRP causes dissociation of the ET-1/ET-A complex was the aim of a study published this year, which showed that termination of the ET-1 binding to the ET-A receptor by CGRP requires specific downstream signalling of the G protein βγ-subunit. This signalling is

cAMP independent because the adenylat cyclase can be activated only by Gα subunit (Meens et al., 2012).

Keeping our observation of the reduced nociception of CLR-tg mice in mind we speculate that the anti-endothelinergic action of CGRP is not limited to smooth muscle cells, but that the same mechanism occurs during the nociception process in the peripheral sensory fibers. CGRP might terminate ET-1 induced pain, as ET-A receptors are present on C-fibers (Pomonis et al., 2001) and CGRP is released from peripheral endings of nociceptors upon stimulation. Thus CGRP could act in an autocrin manner to dissociate ET-1 / ET-A receptor complexes on pain fibers (*Fig. 4*). The expression of CGRP receptors on nociceptors, however, has not yet conclusively been proven because of the lack of good commercial antibodies, but using custom made antibodies good reasons are provided by others suggesting that functional CGRP receptors are indeed expressed on peripheral endings of nociceptors (Lennerz et al., 2008; Cottrell et al., 2005).

The putative role of CGRP in the ET-1 pain axis is illustrated in *Fig. 4*.

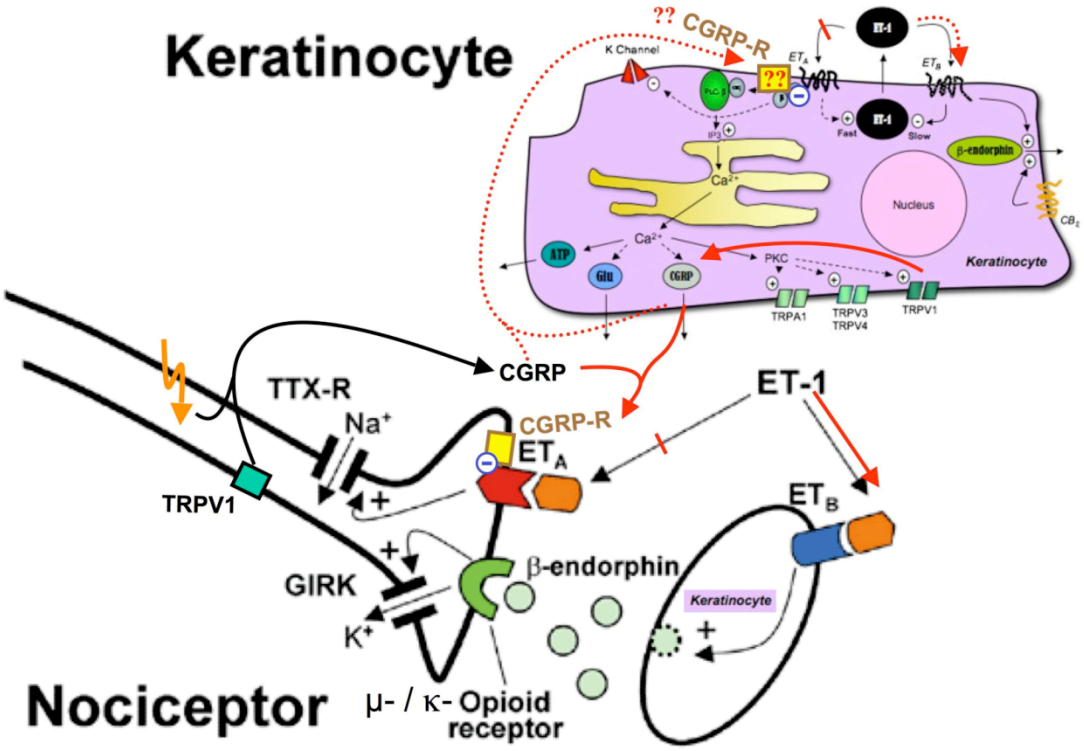


***Figure 4**: Proposed role of CGRP in the ET-1 mediated pain axis (modified from Khodorova et al., 2009a and Khodorova et al., 2003) After a nociceptive stimulus, caused for example by a needle or by burning, the nociceptor depolarizes, releasing CGRP. On the other hand, injured keratinocytes release ET-1, which in turn binds to the ET-A receptor on nociceptors. The ET-1/ET-A complex on nociceptors induces pain sensation. The crosstalk between activated CGRP receptor and ET-A receptor terminates the binding of ET-1 with its receptor, increasing the availability of ET-1 that can bind to the ET-B receptor on keratinocytes, enhancing the release of β endorphin. Whether this interaction between the two receptors occurs also on keratinocytes, depends on the existence of CGRP receptor on cutaneous cells, which has not yet been shown (the red arrows illustrate the hypothesis whereas black arrows are supported by the literature)*

1.4.3. Goal of the study

The purpose of this dissertation is to understand the role of CGRP in the ET-1-mediated pain perception at the level of the peripheral nociceptive fibers, namely at the first step of the perception of pain. The hypothesis that CGRP acts as a peripheral analgesic factor, stopping the ET-1 mediated pain on nociceptors, was tested with two different animal models: i) the CLR-transgene mouse (CLR-tg), that was expected to have a decreased nociception due to the overexpression of CGRP-receptors and ii) the

αCGRP knockout (αCGRP$^{-/-}$) mouse that should have an increased nociception, because without αCGRP the proposed interference of CLR signalling with the ET-1/ET-A complex on nociceptors cannot take place.

The characterization of the nociception was assessed with established experiments in vivo, involving thermal and mechanical sensitivity. Animal paws were stimulated either with hot water (52°C) during anesthesia, or unstrained with radiant heat, or with von Frey filaments. These two latter experiments were performed before and after inducing neuropathic pain through a constriction of the sciatic nerve. The reaction time or the force applied were the parameters used to quantify the thermal or mechanical sensitivity respectively. In addition, licking / biting times after intraplantar injection of ET-1 was measured in the above-mentioned genetically modified mice with or without pretreatment with CGRP or the CGRP-antagonist CGRP8-37.

In vitro study to prove a CGRP-induced dissociation of the ET-1/ET-A complex in the skin was performed according to the method published by Meens et al., 2010.

2. MATERIAL AND METHODS

2.1. Animals and house conditioning

All the mice used for the experiments were kept in a temperature (21°± 2°C) controlled environment with a 12 h / 12 h light-dark cycle. Food and water were available *ad libitum*.

The experimental animals were handled with care and adapted to the experimental conditions before starting the experiments.

All experiments were approved by the Veterinary Office of the Canton Zurich, Switzerland.

2.2. Animal models

In our study we performed the experiments with two different mouse genotypes, the calcitonin receptor-like receptor -transgenic mouse (Kunz et al., 2007) and the αCGRP knockout mouse (Lu et al., 1999). Two wild-type strains were used, because of the different genetic background of the CLR-tg and the $\alpha CGRP^{-/-}$ mice.

2.2.1. CLR-transgenic mouse

Calcitonin receptor-like receptor (CLR) is essential to survive, indeed mice lacking the CGRP receptor die between day 13.5 and 14.5 of gestation (Dackor et al., 2006). Hydrops fetalis, thin vascular smooth muscle walls, small and abnormally developed heart and general cardiovascular failure are the causes of the lethality of the $CLR^{-/-}$. This phenotype is highly similar to that observed for the adrenomedullin knockout mice (Caron & Smithies, 2001).

The CLR-transgenic (CLR-tg) mouse model is an established transgenic mouse line, which is characterized by the overexpression of the CLR in neurons, e.g. the sympathetic postganglionic neurons. The overexpression of the v5-tagged rat CLR is under the control of a mouse smooth muscle α-actin promotor (Ittner et al., 2008;

Kunz et al., 2007; *see Fig. 5*). Subjective observations revealed a decreased nociception in the transgenic mouse line compared to the wild-type littermates.

Phenotype:

- Alopezia and irregular hair growth, visible two weeks after birth.
- Acute angle closure glaucoma (Ittner et al., 2008)
- Growth retardation
- Sustained tachycardia after intravenous injection of CGRP (Kunz et al., 2007)

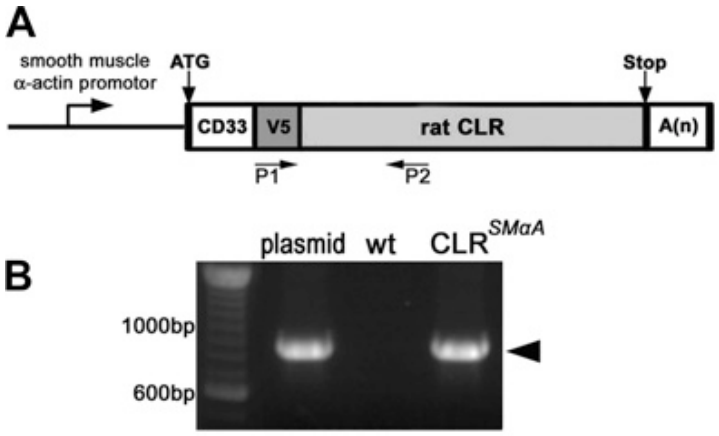


***Figure 5**: Generation of V5 rat calcitonin receptor-like receptor (CLR) transgenic mice. A: the transgene consists of a smooth muscle α-actin promoter, a DNA fragment encoding the signal sequence of the CD33 protein (CD33), a V5 epitope-tag (V5), the cDNA of the rat CLR, and the polyadenylation signal of the bovine growth hormone gene [A(n)]. P1 and P2 are the position of forward and reverse primers used for genotyping the mice. B: agarose gel electrophoresis of PCR-amplified DNA from mouse tail biopsies with the predicted 880-bp transgene-derived product (arrowhead) present in CLR transgenic ($CLR^{SM\alpha A}$) mice but not in control (wt) littermates (modified from Kunz et al., 2007)*

2.2.2. αCGRP-knockout mouse ($\alpha CGRP^{-/-}$)

Since the knockout of both alpha and beta CGRP is lethal, an $\alpha CGRP^{-/-}$ mouse was created in 1999 (Lu et al., 1999). An important detail of this mouse is the ablation of αCGRP, but not of calcitonin, which is in fact the product of an alternative splicing of the RNA encoding for CGRP and calcitonin (Gangula et al., 2000). Lu et al. used a

special strategy, because the classical knockout of the whole gene would result in lacking not only the αCGRP peptide, but also the calcitonin. To avoid this, and misinterpretations of phenotypic results, a stop codon (TGA) was introduced before the αCGRP-encoding region, in exon 5. This modification results in the loss of expression of αCGRP and of a 4-aminoacid Peptide (DLQA), deriving from the carboxyl-terminus of the αCGRP prohormone. Because the real function of this 4-aminoacid peptide is not known, the targeting vector was further modified and an additional copy of this peptide was inserted (Lu et al., 1999; *see Fig. 6*).

The resulting αCGRP$^{-/-}$ is phenotypically indistinguishable from the wild-type littermates.

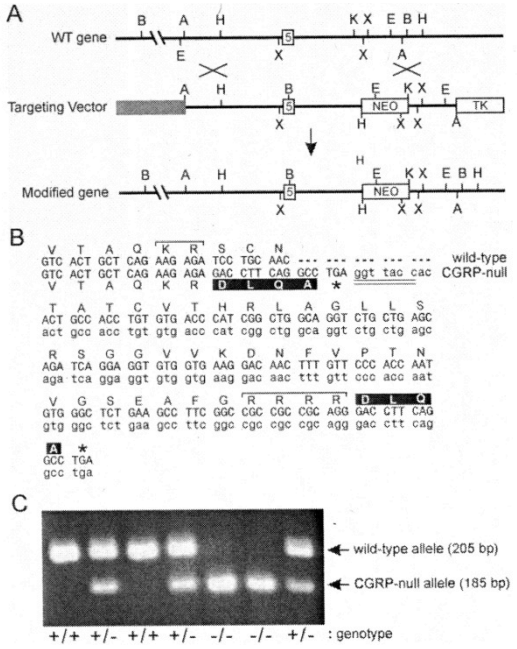


***Figure 6**: Targeting strategy for the generation of αCGRP$^{-/-}$ mice. (A) Schematic diagram and restriction map of the mouse calcitonin / αCGRP gene before and after targeted gene modification. (B) Nucleotide and amino acid sequence comparison between the wild-type and the αCGRP$^{-/-}$ (αCGRP-null) alleles within the coding region of exon 5. Introduction of a stop codon (*) immediately upstream of the CGRP coding region and duplication of the predicted 4-amino-acid carboxyl-terminal peptide (DLQA). (C) Analysis of mouse genotype by polymerase chain reaction amplification of mouse tail genomic. The migration positions for the wild-type (+/+; 205 bp) and αCGRP-null (-/-; 185 bp) alleles are indicated as is the determined mouse genotype (Lu et al., 1999)*

2.3. Assessment of nociception

2.3.1. Thermal Stimulation in etomidate anesthesia and c-fos expression in the spinal cord

Systemic responses to noxious stimuli are provoked by the activation of the sympathetic nervous system and can be quantified by an increase of the blood pressure and of the heart rate (Lamont et al., 2000). 10 to 12 weeks old female CLR-tg and αCGRP$^{-/-}$ mice and their respective control mice were subjected to thermal stimulation in etomidate anesthesia and blood pressure and heart rate were monitored simultaneously. Two hours later the spinal cord was isolated and c-fos immunoreactivity level was determinded. This latter is an established marker for neuronal activation and nociceptor stimulation (Munglani & Hunt, 1995; Hunt, 1987; Harris, 1998; Morgan & Curran, 1991; Morgan & Curran, 1989). In addition, the effect of pretreatment with 20 µmol/kg αCGRP8-37 (Bachem, Switzerland) was tested. The CGRP antagonist was injected intravenously 25 min after starting the etomidate anesthesia and 5 min prior to the stimulations. Only the CLR-tg line was subject of a pretreatment with the antagonist, because in αCGRP$^{-/-}$ mice it cannot have an effect.

2.3.1.1. Thermal Stimulation in etomidate anesthesia

The mice were initially anesthetized with isoflurane (Attane™ , Provet, Switzerland) in dorsal recumbency. A catheter was placed into the carotid artery in order to measure the heart rate and the blood pressure. Another catheter was placed into the external jugular vein, to permit the switch from the isoflurane anesthesia (gas) to the etomidate (Etomidat Lipuro, B. Braun, Germany, 2mg/ml) anesthesia (infusion). Changing anesthesia is very important, because Isoflurane causes a dose-dependent depression of the cardiovascular system, whereas the influence of etomidate on the cardiovascular system is minimal (drug compendium, Switzerland). 30 minutes after the beginning of the etomidate anesthesia (7.5-10µl/min) the experiment could start with the immersion of the left hind paw (till the ankle joint) in 52°C hot water for 20 seconds, 10 times, with an interval of 90 seconds between the stimulations. In every single animal we could observe an increase of the blood pressure and of the heart rate

temporally related to the thermal stimulus. The mean increase of the systolic pressure and the heart rate during stimulations was used for statistical analysis.

2.3.1.2. Tissue perfusion

After thermal stimulation, the mice were kept in anesthesia for another 2 h prior to perfuse them with the aim to fix the tissue. Two hours is the time needed for significant c-fos expression in the spinal cord (Harris, 1998).

After reintroduction of isoflurane anaesthesia (5% in 100% oxygen), the mice were perfused transcardially with ice-cold 0.1 M phosphate buffer (PB) (*see Tab. 1* for composition of the solution), followed by 4% paraformaldehyde in 0.1 M PB. The spinal column was removed with the spinal cord in place and the whole backbone was immersed overnight in 4% paraformaldehyde in 0.1 M PB. The spinal cord was then stored in 20% sucrose in 0.1 M PB for 48-72 h at 4°C to ensure cryoprotection. The tissue was then frozen and kept at -20°C until slicing was done.

Table 1: *0.1 M phosphate*

	Substance	**g/L**	**End concentration**
Stock 1	$NaH_2PO_4 . H_2O$	27.8	0.2 M
Stock 2	$Na_2HPO_4 . 2H_2O$	35.6	0.2 M
To obtain 0.2 M PB 95 ml of Stock 1 was mixed with 405 ml of Stock 2. For the 0.1 M PB solution, 0.2 M PB was diluted 1:1 with distilled water. The pH was checked by cold solution and adjusted to 7.2			

2.3.1.3. Slicing and c-fos staining

The spinal cord together with the spinal column, was sliced into 20µm thick sections with a cryostat (CM 3050, Leica, Germany) and thaw-mounted on microscopic glass slides (Superfrost® Plus, Gerhard Menzel GmbH, Germany). The c-fos expressing cells in the spinal cord were visualized using an immunhistochemical procedure as described previously (Riediger et al., 2004), with some modifications. Briefly, slides that were stored at -20°C were dried 1 h at room temperature. Then the slides were re-hydrated and washed in Phosphate Buffer Saline (PBS, pH 7.4) with 0.1 % Triton X-

100 (PBST 0.1%: 0.1 ml Triton X-100 in 100 ml PBS) 2 x 5 min. To block unspecific binding, slides were incubated in a solution containing 1.5% normal donkey serum (Jackson, 017-000-121) in PBST 0.3% and a 1:5 avidinD solution (Avidin/Biotin Blocking Kit, Vector Laboratories, U.S.A.) for 2 h at room temperature. After a rinsing step in PBST 0.1% of 30 min (3x10 min), sections were incubated for 48 h with rabbit anti-fos antibody (Calbiochem BC 38) 1:5000 in PBST 0.3% and 1:5 biotin solution (Avidin/Biotin Blocking Kit, Vector Laboratories, U.S.A.) at 4°C. To remove unbound antibody, slides were rinsed 5 x 10 min in PBST 0.1%. Sections were then incubated with the secondary biotinylated donkey anti-rabbit IgG antibody 1:500 (Jackson, 711-065-152) in PBST 0.3% and 1.5% normal donkey serum for 90 min. Slides were washed for 30 min in PBST 0.1% and then incubated for 1 h in avidin-biotin-peroxidase complex (1:100 in 0.3% PBST, Vectastain ABC kit, LINARIS GmbH, Dossenheim, Germany). After that, slides were rinsed 10 min in PBST 0.1% and 10 min in Tris(hydroxymethyl)aminomethane-hydrochlorid acid (Tris-HCl 0.05M, pH 7.6). DAB was used to detect the c-fos immunoreactivity (c-fos-IR). The solution was prepared with 0.05% DAB, 0.009% H_2O_2, 0.04% $NiCl_2$ and 0.08% $CoCl_2$ in Tris-HCl and the section were stained for 8 min. Afterwards, slides were rinsed again for 5 min in TrisHCl 0.05M and 2 x 5 min in PBST 0.1%. The final step was dehydration of the sections in graded alcohol (50%, 75%, 95%, 100%) and incubation in xylol (5 min in each solution). At the end sections were coversplipped with entellan (Merck KGaA, Darmstadt, Germany). C-fos-IR was detected with a light microscope (Axioscop 2, Carl Zeiss AG, Switzerland).

Stained cells in the dorsal horn of the spinal cord (Lamina I and Lamina II of the lumbar spinal cord, left side, according to Rexed's classification (Rexed, 1952)) were counted. For the statistical analysis the mean value of cells per section per animal was counted (n = number of animals).

2.3.2. Assessment of the mechanical and thermal sensitivity before and after chronic constriction of the sciatic nerve

These experiments allowed the assessment of mechanical and thermal sensitivity of a mouse hind paw in healthy state (baseline values) and in a condition of neuropathic

pain. 6-8 weeks old male mice, of the genotype αCGRP$^{-/-}$ and CLR-tg, were tested on their mechanical and thermal sensitivity. After evaluation of baseline values, a unilateral constriction of the sciatic nerve was performed. Thermal and mechanical sensitization was assessed 7 days later.

To be aware of the behavioural state of the animals is an essential point to avoid incorrect conclusions. It is important that all the stimulations are done when the animal is in a "resting" behavioural state, meaning that the mouse is calm and the eyes are open or half-open, because the trend of grooming or deep sleeping animals is to be hypoalgesic to nociceptive stimuli (Callahan et al., 2008).

2.3.2.1 Mechanical stimulation

The mice were placed under a transparent plastic box (4x4x7 cm) on an elevated metal mesh floor. The animals were unrestrained and the time of habituation was ca. 45 minutes, until the mice were sitting and calm. A dynamic von Frey filament (IITC, Woodland Hills, USA) was used to assess the paw withdrawal threshold in grams, namely the minimal pressure required to achieve 100% response. The von Frey hair was inserted vertically from below, through the holes of the mesh floor, and the plantar side of the hind paws was stimulated. The paw withdrawal threshold was registered when the mouse was responding to the stimulus with a flicking. At least 5 readings were taken per animal and paw. For the statistical analysis the mean paw withdrawal threshold of an individual animal was used (n = number of animals).

2.3.2.2. Thermal Stimulation

The thermal sensitivity was assessed with the plantar test apparatus (Ugo Basile, Italy). Also in this experiment the mice were placed under a transparent plastic box and the essay started only when the animals were calm and habituated to the new environment. A radiant infrared light source was applied on the plantar side of the hind paws and the time to the flicking reaction was registered as the paw withdrawal latency, to the nearest 0.1 seconds. At least 5 measurements were performed per animal and paw. For statistical analysis the mean paw withdrawal latency of an individual animal was used (n = number of animals).

2.3.2.3. Chronic constriction of the sciatic nerve (CCI)

The CCI was performed unilaterally as described previously (Bennett & Xie, 1988), with some modifications. The mice were anesthetized with Isoflurane and a latero-dorsal access to the sciatic nerve was performed. While exposing the nerve from the rest of the tissue, care was taken to avoid touching and damaging it, and thus influence the outcome of the constriction. Three ligatures were tied loosely around the sciatic nerve, ca. 1 mm distance from each other (Silkam® 5/0, Braun; *see Fig. 7*).

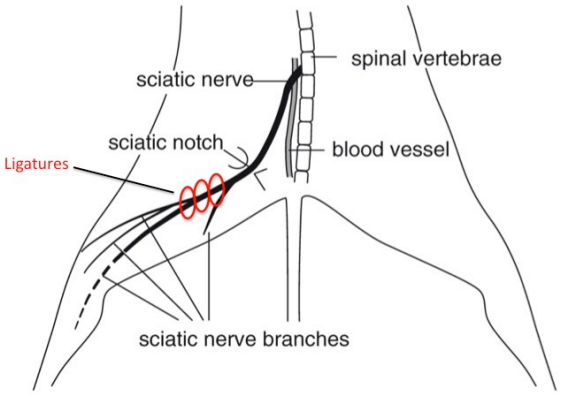


***Figure 7**: Anatomy of the sciatic nerve in rodents (modified from Weinstein & Wu, 2001)*

At the end of the surgery the skin was sutured.

2.3.3. Licking-biting time after intraplantar endothelin-1 injection

Intraplantar injection of endothelin-1 (ET-1) induces mechanical allodynia in rodents (Baamonde et al., 2004; Balonov et al., 2006; da Cunha et al., 2004). According to our hypothesis CGRP released from the nociceptors inhibits the binding of ET-1- to the ET-A receptor and therefore should reduce the ET-1 mediated sensation of pain (*see introduction*). Thus, this experiment was essential to demonstrate the interaction between the different peptides and receptors.

A study published by Khodorova et al. (Khodorova et al., 2009b) shows that the late phase of allodynia (60-90 min after ET-1 injection) is attenuated by the intraplantar further or co-administration of CGRP8-37 in a dosage of ca. 20 nmol/paw, corresponding to a 100-times higher antagonist amount compared to our dosages. We

were using intentionally a lower antagonist amount because at higher concentrations the peptide could cross the Blood Brain Barrier (BBB) and thus effects in the spinal cord could cover the pro-algesic effect of CGRP8-37 in the periphery.

Suggestions for the injection protocol were taken from the literature (Motta et al., 2006; Khodorova et al., 2003, Khodorova et al., 2009b). Four to five months old male CLR-tg and αCGRP$^{-/-}$ mice were anesthetized with sevoflurane (Sustane Sevoflurane, Provet AG, Switzerland). As pretreatment either 0.2 nmol CGRP (Rat, Sigma-Aldrich, 099K4781, Switzerland), 0.2 nmol CGRP8-37 (Bachem, Switzerland) in 200 µl PBS or only the vehicle PBS (200 µl) were injected subcutaneously into the back skin (pretreatments drugs were injected subcutaneously and not intravenously, in order to elicit a lower peak concentration in plasma, but a prolonged peptide action). After waiting 7 min, the intraplantar injection of the left hind paw with ET-1 (Endothelin-1, Sigma-Aldrich, 117399-94-7, Switzerland) was performed as described in (Khodorova et al., 2003). 10 µl of a 40µM ET-1 solution (400 pmol/paw) were gently injected with a Hamilton Microliter ™ Syringe 100 µl (Nevada, U.S.A.) coupled to a 30-gauge needle. The needle was inserted ca. 2-3 mm under the plantar skin and the paw was cooled with ice 2 min before and 1 min after the injection, to avoid a too fast diffusion of ET-1 into the systemic circulation.

Then the anesthesia was stopped and the mice were observed for 75 min after ET-1 injection in a calm and dim room. The average time spent with spontaneous licking and biting the injected paw minus the non-injected paw was used for statistical analysis.

2.3.4. Verification of RAMP1- and CLR-RNA expression on keratinocytes

In our hypothesis we assume that keratinocytes express functional CGRP receptors. Because this has not yet been shown, we searched for the presence of CGRP receptors on a ribonucleic acid (RNA) level. RNA of epidermal keratinocytes was isolated and reverse transcripted in complementary desoxyribonucleic acid (cDNA). A polymerase chain reaction (PCR) with specific primers for RAMP 1 and CRLR was performed (Kroeger et al., 2009; *see Tab. 2*), the product was loaded on a 2% agarose gel and an agarose gel electrophoresis was run to visualize the specific bands.

2.3.4.1. Isolation of keratinocytes

Adult wild-type (BL6) mice were euthanized (CO_2 overexposure). The hairs on the back were shaved and the skin was washed several times at first with an iodine solution and then with a 70% ethanol solution. The hairless part of the back skin (epidermis plus dermis) was removed with surgical scissor and forceps and the fat of the subcutis was scraped away with a scalpel, paying attention to not damage and perforate the skin. The back skin was placed in a petri dish filled with 4-5 ml of 0.8% trypsin in Dulbecco Modified Eagle Medium (DMEM™, Gibco®, U.S.A.), dermal side down, taking care that the edges were stretched and that the skin was floating on the solution surface. After 1 h incubation at 37°C (continuously shaking), the epidermis (shiny, thin and transparent) was separated from the dermis (red, relatively thick and opaque). The epidermis was then placed in a 15 ml falcon tube, filled with 5 ml 0.00025% DNase I (0.0125g DNase in 50 ml DMEM, Gibco®, U.S.A.) and incubated for 30 min at 37°C. This last incubation had the goal to separate the keratinocytes from each other. After all, the DNase solution with the keratinocytes was filtered through a 70 µm cell strainer and centrifuged at 1500rpm for 5 min. The cell pellet was then re-suspended in 1 ml TRIzol® Reagent (Invitrogen, U.S.A), and the RNA isolation was performed immediately after, to avoid RNA degradation.

2.3.4.2. RNA Isolation with TRIzol® reagent

Before starting with the RNA isolation, the environment and the working tools were cleaned with RNase ZAP™ (Invitrogen, U.S.A). Only RNase-free pipet tips and eppendorf tubes were used. Gloves were often changed and probes were handled meticulously, to prevent RNase contamination or RNA degradation.

TRIzol® reagent is a solution containing phenol and guanidine isothyocyanate, which maintains RNA-integrity after lysing the cells with a homogenizer. The RNA was isolated following the protocol provided by the manufacturer. Briefly, 1 ml of TRIzol® Reagent was added to the cells pellet, the cells were disrupted by pipetting up and down several times or with a homogenizer and then incubated for 5 min at room temperature. 200 µl RNase-free chloroform were added for the separation of the phases. After mixing strongly by hand and an incubation time of 2-3 min at room temperature, the homogenate was centrifuged for 15 min at *12'000 x g* at 4°C. Three

different phases were then recognised: a lower red organic phase, containing phenol and chloroform, an interphase, and an upper transparent aqueous phase.

The aqueous phase, containing the RNA, was transferred to a new tube, pipetting very carefully, to avoid contamination with the organic phase.

The RNA was precipitated by adding 500 µl of 100% RNase-free isopropylalcohol, incubating 10 min at room temperature and centrifuging for 10 min at *12'000 x g* at 4°C. The supernatant was removed and the pellet washed with 1 ml 75% ethanol (ethanol dissolved in RNase-free water) and centrifuged again for 5 min at *12'000 x g* at 4°C. The wash was discarded and the RNA pellet air-dried for 5-7 min.

Finally, the pellet was re-suspended in RNase-free water (ca. 30 µl) and heated at 60°C for 5 min.

The RNA concentration was analyzed with the spectrophotometer NanoDrop 2000 (Thermo Scientific, USA), before reverse transcription.

2.3.4.3. Reverse Transcription

The reverse transcription of RNA in cDNA was performed with the RNAqueous®-4PCR Kit (Ambion®, U.S.A).

2000 ng RNA were annealed with 2 µl oligonucletide primer (Oligo dT) and the total volume of 12 µl was filled up with RNase-free water. The sample was then mixed, briefly spinned and incubated for 3 min at 70-85°C. The remaining components were added to achieve a total volume of 20µl: 2µl 10x RT buffer, 4µl dNTP mix, 1µl RNase inhibitor and 1µl MMLV-retrotranscriptase.

After 1 h incubation at 42-44°C the sample was heated at 92°C for 10 min to inactivate the reverse transcriptase. cDNA was stored at -20°C.

2.3.4.4. Polymerase chain reaction

cDNA was amplified with specific CGRP receptor primers (*see Table 2*) with polymerase chain reaction (PCR).

For every PCR reaction the following components were added to 2µl cDNA (200 ng):

- 2.5 µl CRLR fwd primer (10µM)
- 2.5 µl CRLR rev primer (10µM)
- 2.5 µl dNTP's (2.5mM, Sigma-Aldrich, Switzerland)
- 2.5 µl 10x PCR Buffer (Sigma-Aldrich, Switzerland)
- 12.5 µl distilled water
- 0.5 µl Taqman DNA polymerase (Sigma-Aldrich, Switzerland)

The mixture was then placed in a thermocycler and the PCR-cycle was set as following: 95°C 1min – 40 x (95°C 30sec – 58°C 20sec – 72°C 40sec) – 72°C 5min

In the mean time 1 g of agarose was dissolved in 50ml Tris-acetate-EDTA buffer (TAE Buffer) by heating them in a microwave. The gel was cooled down for few minutes, before 1 µl of ethidium bromide (EtBr) was added, an agent that intercalates with DNA and fluoresces by UV-light exposure. The gel was poured into an electrophoresis chamber and waited till it was solid.

At the end of the PCR reaction, the PCR product was mixed with a loading dye and 15µl were loaded on the gel. The electrophoresis was run for 1 h with a voltage of 60 volts. Bands were visualized in a UV-chamber.

***Table 2**: Primer sequences for RAMP1 and CRLR subunits (Kroeger et al., 2009)*

	Sequence 5'-3'	**Product size (bp)**
RAMP1 forward	GACGCTATGGTGTGACT	249
RAMP1 reverse	GAGTGCAGTCATGAGCAG	
CRLR forward	GGTACCACTACTTGGCATTG	262
CRLR reverse	GTCACTGATTGTTGACACTG	

2.3.5. Staining and de-staining of mouse skin with ET-1

As described by Meens et al. (Meens et al., 2009) CGRP is able to relax ET-A mediated contracted rat mesenteric arteries. Because we think that also in the skin CGRP can terminate the ET-1/ET-A interaction, we repeated the experiment done by Meens et al. with some modifications. To get familiar with the technique and to test the functionality of the peptides, we repeated the experiment also with rat mesenteric arteries as described by Meens et al. (no statistical analysis). Our results confirmed those of Meens et al., demonstrating that the experiment works. Indeed, as we can see in *Fig. 8*, the fluorescence level increases after incubation with RhET-1 and decreases after incubation with CGRP. The initial incubation with BQ788 (ET-B receptor antagonist) has the goal to limit the RhET-1 binding to the ET-A receptor.

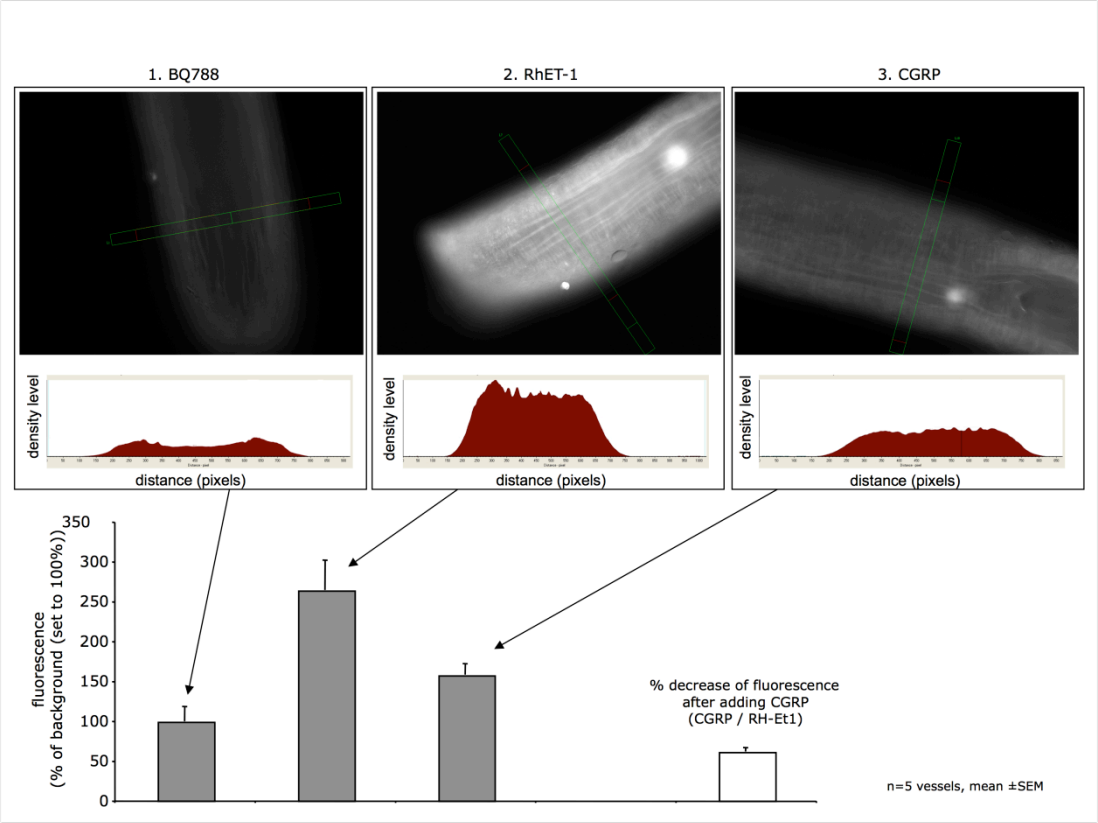


***Figure 8**: Representative photomicrograph of fresh rat mesenteric arteries after successively incubation with BQ788, RhET-1, and CGRP. The graph represents the fluorescence changes of the vessels in per cent of background.*

Plantar paw skin of euthanized mice was frozen, sliced 10µm thick with a cryostat (CM 3050, Leica, Germany) and thaw-mounted on microscopic glass slides (Superfrost® Plus, Gerhard Menzel GmbH, Germany).

Following working solutions were prepared:

- 100µM ET-B receptor antagonist (BQ788, Sigma-Aldrich, Switzerland) in HEPES buffer

- 16nM RhET-1 in HEPES buffer (RhET-1 was a kind gift of M. Meens, Maastricht University, Netherlands)

Two groups of slides were created with different incubation protocols *(see Tab. 4)*. The incubation time for each peptide was 10 min at 37°C. RhET-1 fluorescence was detected with a light microscope (Axioscop 2, Carl Zeiss AG, Switzerland). After

each incubation photomicrographs were taken using a digital camera (AxioCam, Carl Zeiss AG, Switzerland). Between the different incubation steps, slides were washed in HEPES buffer *(see Tab. 3)* for 3 min. The fluorescence intensity was quantified using an image analyzing system (MCID Analysis 7.0, Ontario, Canada). Background fluorescence was defined by taking a photomicrograph of the subcutis before the incubation with RhET-1. In the first group (group 1) sections were incubated with BQ788, followed by RhET-1 and finally by CGRP. The goal of the second group (group 2) was to evaluate if the time of incubation and the washing steps influenced the fluorescence density.

***Table 3**: Components of HEPES buffer. The pH was set to 7.4*

Substance	**Concentration (mM)**
NaCl	144
KCl	4.7
$CaCl_2$	2.5
$MgSO_4$	1.2
KH_2PO_4	1.2
HEPES	14.9
Glucose	5.5

***Table 4**: Incubation protocols (3 slides per group)*

Group 1	BQ 788	10 min
	RhET-1	10 min
	CGRP	10 min
Group 2	BQ788	10 min
	RhET-1	10 min
	BQ788	10 min

2.4. Statistical analysis

All data are expressed as mean ± SEM. Statistical significance was determined by parametric t-test of data with normal distribution (Kolmogorov-Smirnov normality test) or the Wilcoxon signed rank test, using Prism Version 5.0a for Mac OS X (GraphPad Software Inc., U.S.A.). Differences between two groups were considered significant for p values < 0.05.

3. RESULTS

3.1. Thermal Stimulation in etomidate anesthesia and c-fos expression in the spinal cord

The goal of this experiment was to detect if the genotype αCGRP$^{-/-}$ or CLR-tg has an influence on the pain-induced sympathetic reaction and c-fos expression.

In this experiment the sympathetic reaction following a painful thermal stimulus was quantified with measurements of the increase of systolic blood pressure and heart rate. Statistical analysis was performed using the difference between the systolic pressure and the heart rate immediately before and directly after the stimulus (for detailed description of the evaluation method *see Appendix 1*). The values correspond to the average of 10 stimulations per mouse. In addition, the thermal pain sensitivity was further investigated with the counting of c-fos-positive nuclei in Lamina I and II of the lumbar spinal cord after stimulations. The contralateral side of the stimulated paw was used as negative control, because no c-fos positive cells were expected on this side. *Fig. 9* is a representative image of the lumbar spinal cord of a thermal stimulated mouse. Numerous c-fos positive cells are visible on the ipsilateral side, whereas no such cells can be detected on the contralateral side.

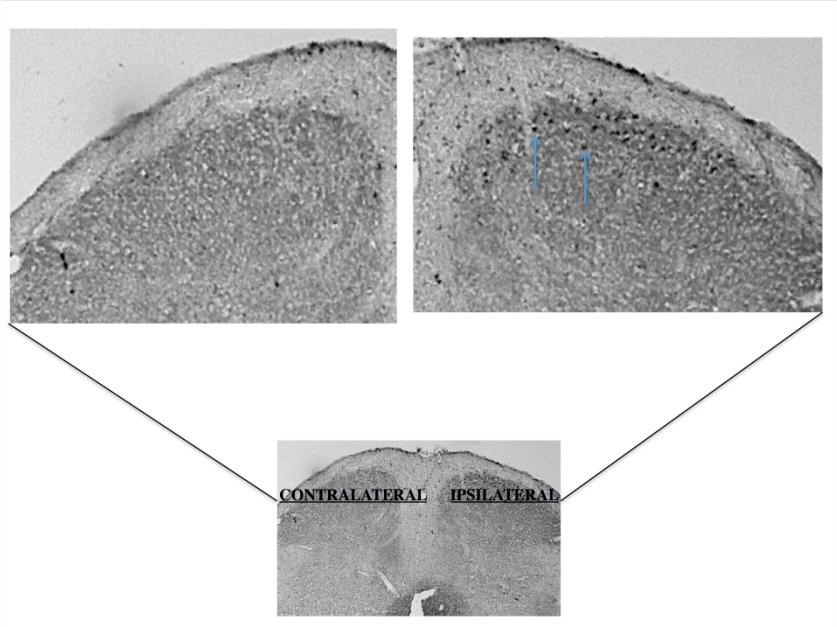


***Figure 9**: Representative immunohistochemistry of c-fos-positive nuclei in the Lamina I and II of the dorsal horn of the lumbar spinal cord. The side of the stimulated hind paw matches with the side of the dorsal horn where c-fos-positive cells are detected. No c-fos-positive cells are observed in the contralateral side. Blue arrows indicate c-fos-positive nuclei*

3.1.1. αCGRP$^{-/-}$ mice

αCGRP$^{-/-}$ (n=5) and control mice (n=5) showed an increase in the mean systolic blood pressure and heart rate after noxious thermal stimulus (no reaction would mean a mean systolic blood pressure difference of 0 mmHg). The mean systolic blood pressure increase of the αCGRP$^{-/-}$ mice was 31.9 ± 3.7 mmHg (mean ± SEM), while of the control group it was 20.9 ± 2.4 mmHg. This significant higher sympathetic response to a noxious stimulus was also seen by an increase of the heart rate: αCGRP$^{-/-}$ mice showed an increase of 36.1 ± 5.9 bpm (mean ± SEM), whereas control mice only 13 ± 1.1 bpm. In contrast, no genotype effect could be observed in the number of c-fos-positive cells. All the results are summarized in *Fig. 10*.

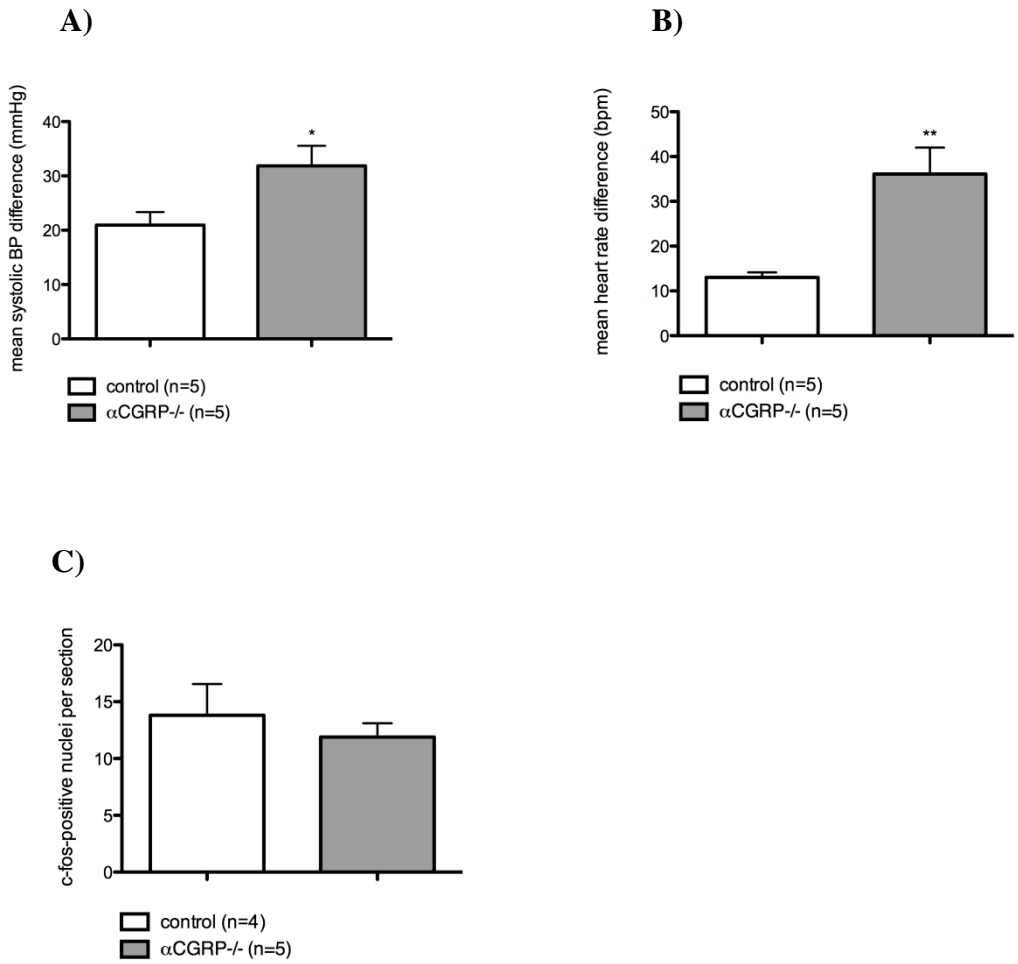


***Figure 10**: A) Mean systolic blood pressure (BP) and* B) *mean heart rate (HR) difference during thermal stimulation in etomidate anaesthesia (in beats per minute, bpm) and* C) *c-fos-positive nuclei per section. All data are expressed as mean ± SEM. The symbols denote significant differences between the two genotypes.* $^{*}p<0.05$; $^{**}p<0.01$

3.1.2. CLR-tg mice

Fig. 11 summarizes the results of the sympathetic reaction during thermal stimulation and the c-fos-positive cells per section after perfusion in CLR-tg mice (n=6-7). These animals showed a mean systolic blood pressure increase of 19.7 ± 2.9 mmHg (mean ± SEM), which is significantly lower compared to the 43.5 ± 3.2 mmHg of their non-transgenic littermates (n=6-7). The decreased nociception of the CLR-tg is also reflected by a lower heart rate increase of 72.1 ± 20 bpm compared to 166.3 ± 18.1 bpm of the wild-type littermates. Regarding the number of c-fos-positive nuclei per section, the control group had a mean of 34.8 ± 1.9 positive cells (mean ± SEM), whereas the CLR-tg mice 16.8 ± 1.1.

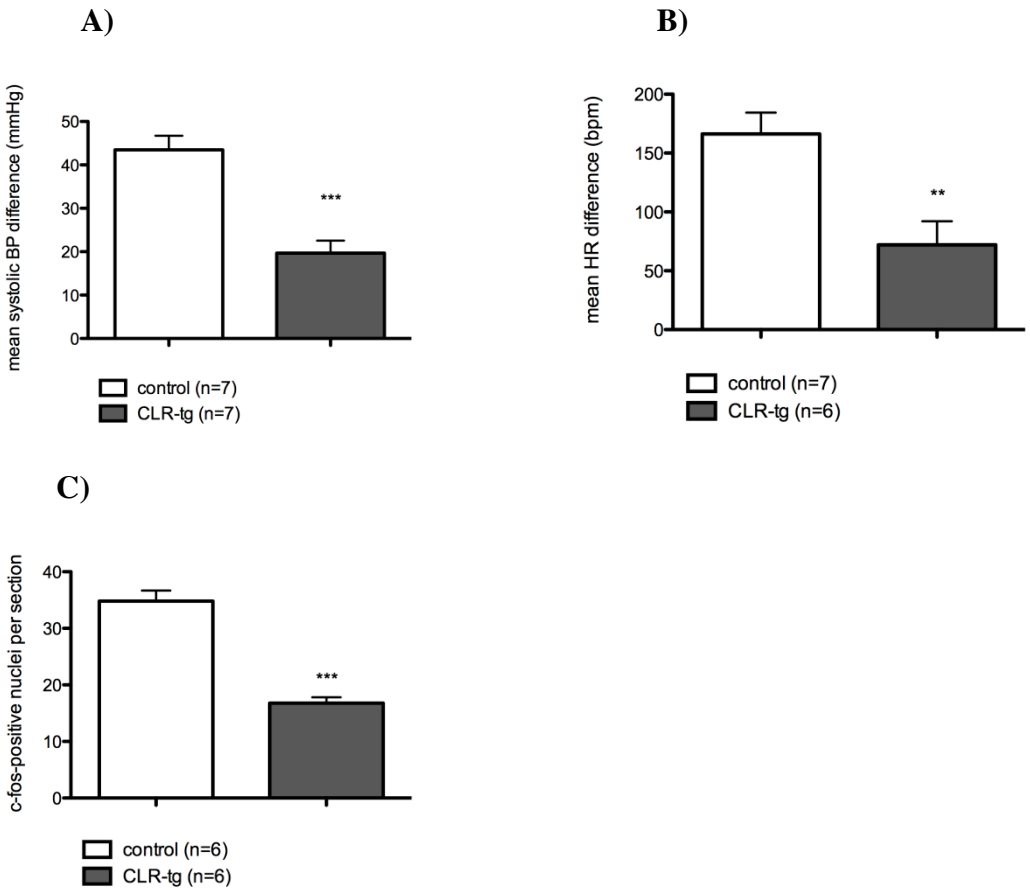
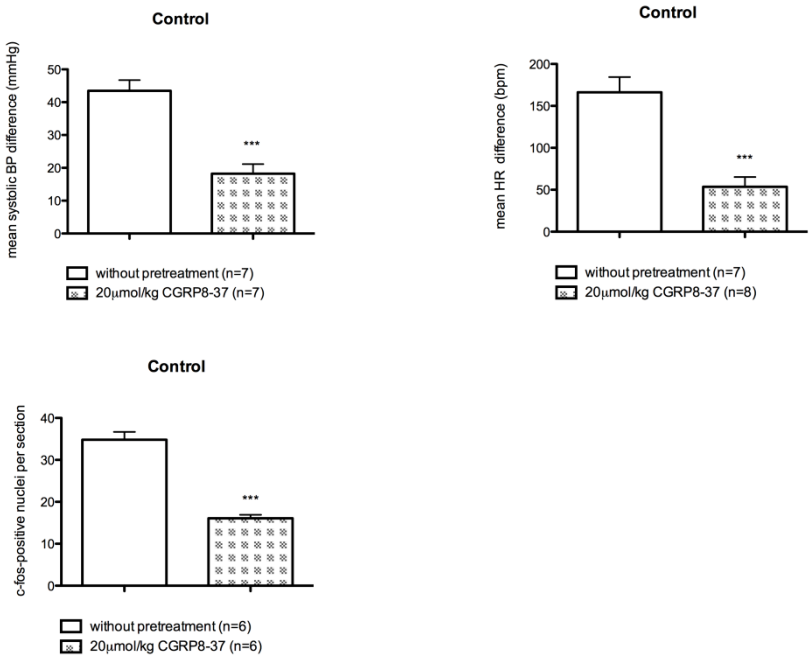


***Figure 11**: A) Mean systolic blood pressure (BP) and* B) *mean heart rate (HR) difference during thermal stimulation in etomidate anaesthesia and* C) *c-fos-positive nuclei per section. All data are expressed as mean ± SEM. The symbols denote significant differences between the two genotypes; $^{**}p<0.01$; $^{***}p<0.0001$*

CGRP8-37 pretreatment

Pretreatment with the CGRP receptor antagonist CGRP8-37 (20µmol/kg) 5 minutes before starting the thermal stimulation is a tool to directly recognize the influence of CGRP on the nociception process. Antagonizing the CGRP receptor, the effect of peripherally and centrally released CGRP during a painful stimulus is counteracted. The graphs in *Fig. 12* show the effect of the pretreatment with 20 µmol/kg CGRP8-37 on the blood pressure, heart rate and number of c-fos-positive nuclei in wild-type mice (A) and in CLR-tg mice (B).

A) Control group



B) CLR-tg group

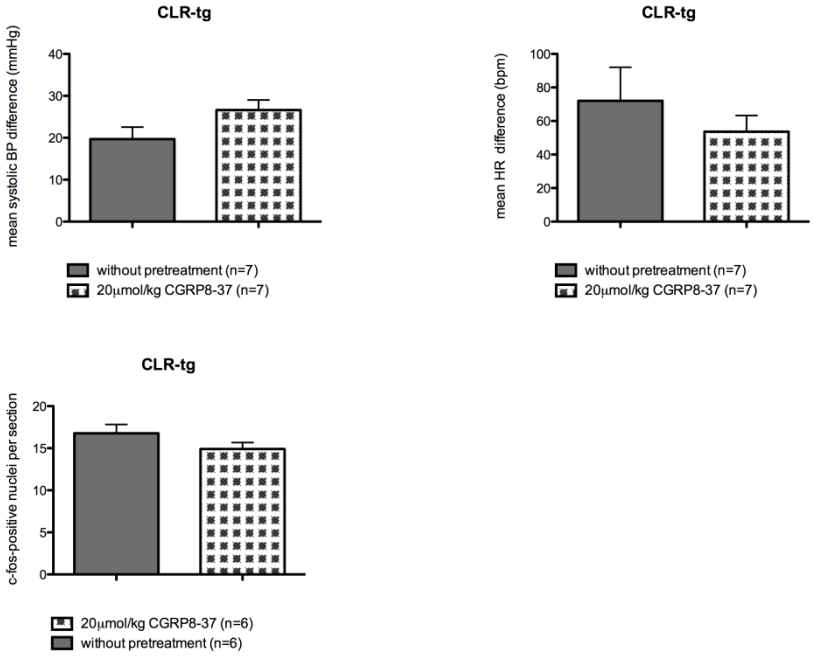


***Figure 12**: A) Effect of CGRP8-37 on BP, HR, and c-fos expression during thermal stimulation in control mice, and B) in CLR-tg mice. All values are means ± SEM. The symbols denote significant differences between non-treated and treated mice: $^{***}p<0.0001$*

An antinociceptive effect of CGRP8-37 was noticeable in the control group (n=6-8). The difference of the mean systolic blood pressure during thermal stimulation was more than 50% compared to the non-pretreated group (43.5 ± 3.2 mmHg (mean ± SEM) without pretreatment and 18.2 ± 2.9 mmHg with pretreatment). Also the heart rate changes of 53.6 ± 11.7 bpm of the antagonized mice were significant lower compared to the HR difference of the non-pretreated group of 166.3 ± 18.1 bpm. The number of c-fos-positive cells confirmed the analgesic action of CGRP8-37 (34.8 ± 1.9 c-fos-positive cells per section in the non-pretreated mice and 16.1 ± 0.8 c-fos-positive cells in the pre-treated group).

In the CLR-tg mice the pretreatment with CGRP receptor antagonist prior to painful stimulation did not result in any analgesic effect. As matter of fact, in terms of mean systolic blood pressure increase, heart rate increase and in the number of c-fos-positive cells, no significant difference between the CLR-tg mice pretreated with CGRP8-37 and the non-pretreated mice could be observed.

3.2. Assessment of the mechanical and thermal sensitivity before and after chronic constriction of the sciatic nerve

These in-vivo experiments had the purpose to understand if the lack of αCGRP or the overexpression of CGRP receptors influences the threshold of thermal or mechanical sensitivity in healthy mice or in mice suffering from neuropathic pain.

Values of thermal and mechanical sensitivity were assessed respectively with the Plantar Test Instrument (Hargreaves Apparatus) and von Frey's dynamic filament, two standardized experimental instruments developed for the study of pain. First, baseline values were collected for each genotype and the respective control groups. After surgery, the ipsilateral side (injuried paw) was compared with the contralateral side (non-injured) within the genotype. Difference score was computed by subtracting the mean latency or withdrawal threshold of the contralateral side from the ipsilateral side.

3.2.1. αCGRP$^{-/-}$ mice

Baseline values

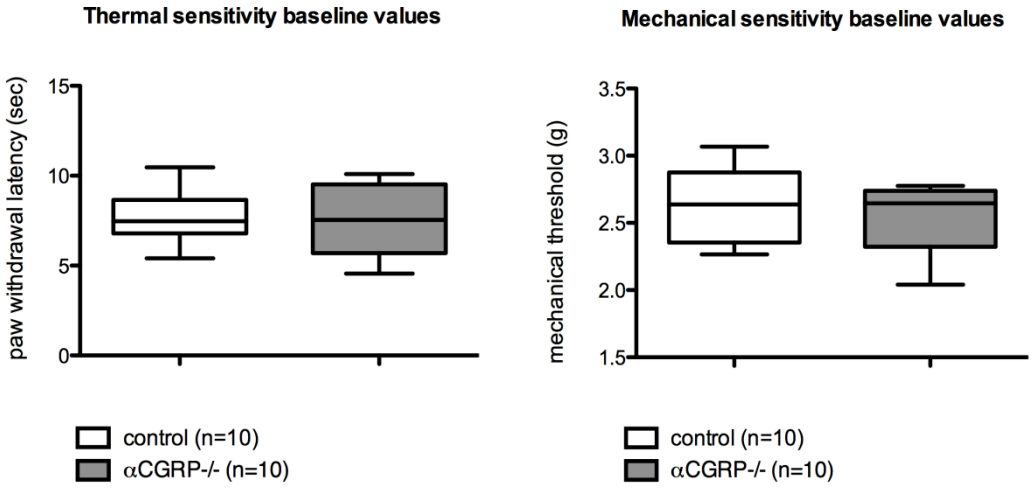


***Figure 13**: Baseline values for thermal and mechanical sensitivity of αCGRP$^{-/-}$ mice compared to the control mice. The whisker plots represent the lower quartile, the median, the upper quartile, and the minimum and maximum value*

In *Fig. 13* two graphs represent the baseline values of mechanical and thermal sensitivity. The values are the same for both groups and no significant difference in mechanical threshold or paw withdrawal latency were observed (n=10 for both groups). The mean paw withdrawal latency of the αCGRP$^{-/-}$ mice was 7.5 ± 0.6 sec (mean ± SEM) and that of the control group 7.7 ± 0.5 sec. During mechanical stimulation the αCGRP$^{-/-}$ mice sensed a pressure of 2.5 ± 0.1 g (mean ± SEM) and the control group 2.6 ± 0.1 g.

Values after CCI

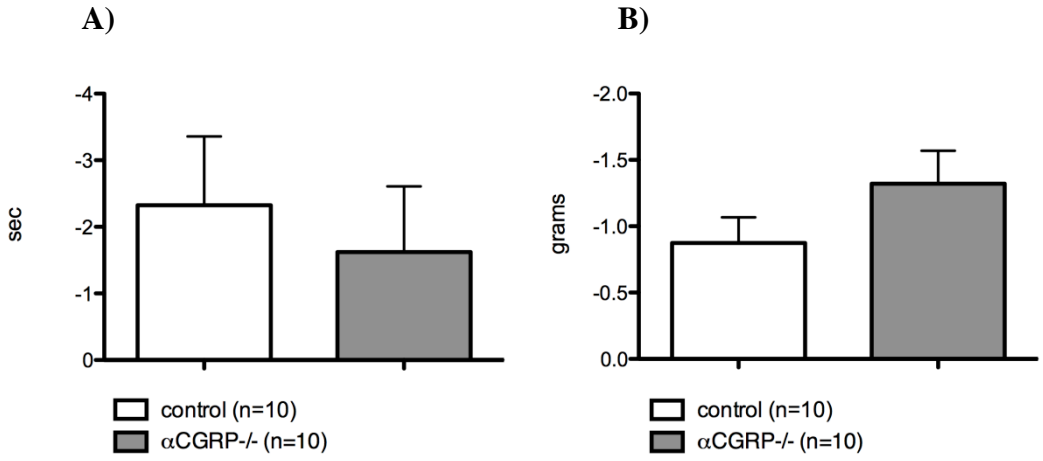


***Figure 14**: Difference score of A) thermal and B) mechanical sensitivity after CCI. All the values are expressed as mean ± SEM (thermal pain: αCGRP$^{-/-}$: -1.6 ± 1.0 sec; wt: -2.3 ± 1.0 sec; mechanical pain: αCGRP$^{-/-}$: -1.3 ± 0.2 g; wt: -0.9 ± 0.2 g)*

The negative difference score indicate a lower threshold of the ligated side. Both groups had a decreased threshold on the ligated side compared to the non-injured side, but no genotype effect regarding the difference score between the two groups of mice could be detected (*see Fig. 14)*.

3.2.2. CLR-tg mice

Baseline values

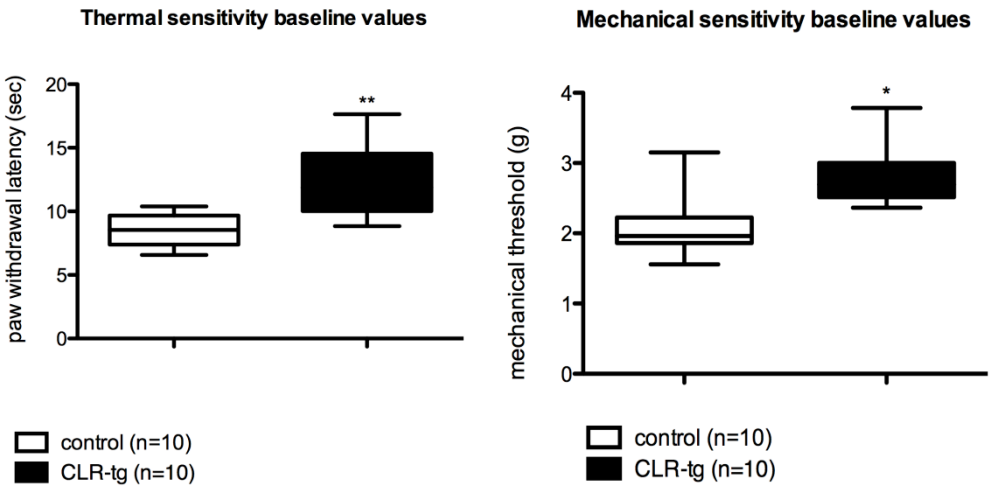


***Figure 15**: Baseline values for the thermal and mechanical sensitivity of the CLR-tg genotype, compared to the wild-type littermates. The whisker plots represent the lower quartile, the median, the upper quartile, and the minimum and maximum value. The symbols denote significant differences between the two genotypes; $^{*}p<0.05$; $^{**}p<0.01$*

Prior to CCI surgery, both thermal and mechanical thresholds of the CLR-tg mice were significantly higher compared to the wild-types (n=10 for both groups). The thermal sensitivity of the CLR-tg mice was 12.2 ± 0.9 seconds and that of the wild-types 8.5 ± 0.4 seconds. The mechanical sensitivity was also decreased in the CLR-tg mice: 2.8 ± 0.2 g, compared to the 2.1 ± 0.1 g of the control mice. These results implicate a genotype effect on the nociceptive threshold in the CLR-tg mice (*see Fig. 15*).

In contrast, after the ligature of the sciatic nerve, the genotype seems not to play a role anymore. Difference scores of the mechanical sensitivity were by tendency lower in the CLR-tg mice (not significant). Regarding the thermal sensitivity, the CLR-tg mice showed a slightly higher difference scores compared to the control mice (*see Fig. 16*).

Values after CCI

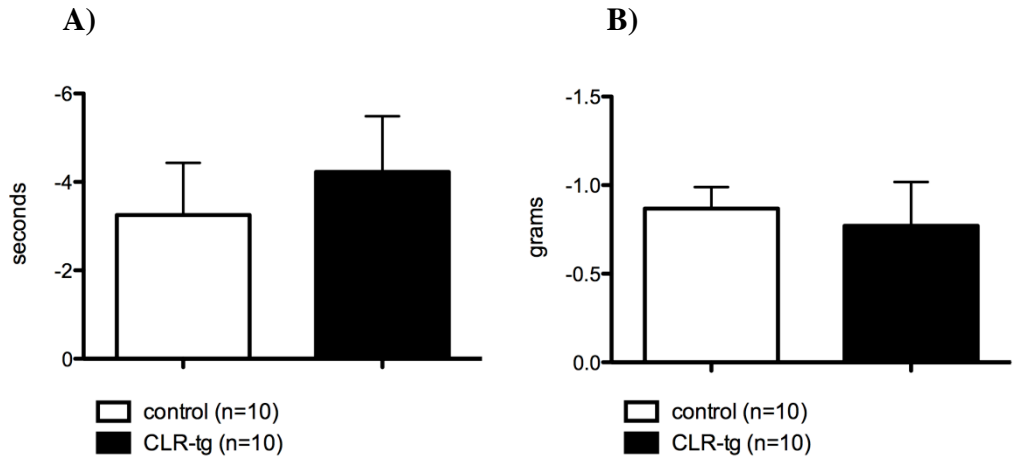


Figure 16*: Difference score of A) thermal and B) mechanical sensitivity after CCI. All the values are expressed as mean ± SEM (thermal pain: CLR-tg: -4.2 ± 1.3 sec; wt:- 3.3 ± 1.3 sec; mechanical pain: CLR-tg: -0.8 ± 0.2 g; wt:- 0.9 ± 0.1 g).*

3.3. Licking / biting time after intraplantar endothelin-1 injection

For this experiment, our purpose was to investigate if the genotype and activation or blocking of CGRP receptors influences the nocifensive behaviour induced by intraplantar administration of ET-1. The modulation of ET-1-induced licking/biting behaviour by CGRP was studied with the administration of CGRP, CGRP8-37 as well as PBS as control prior to the ET-1 injection. Of note, the injected amount of CGRP and CGRP8-37 was considerably lower compared to the dosage used before thermal stimulation during etomidate anaesthesia.

Tab. 5 represents the average time (in seconds) mice spent with licking / biting the injected paw minus the non-injected one, with focus on the different pretreatments.

As only 3-4 mice per group could be measured no statistical analysis had been performed. We observed a decreased licking / biting time of the CLR-tg mice compared to their wild-type littermates (PBS) and an increased duration when pretreated with CGRP8-37. Moreover, wild-type mice (of the CLR-tg genotype) became less nociceptive when pre-treated with CGRP.

The αCGRP$^{-/-}$ mice seem to have an increased nociception compared to their control group and the pretreatment with CGRP decreased in both groups the time spent in licking and biting. Pretreatment of the αCGRP-/- mice with CGRP receptor antagonist increased the licking biting time by only 13.6 sec.

***Table 5**: Shown is the time in seconds mice spent with licking/biting the ET-1 injected paw minus the non-injected paw during 75 minutes after injection. The pretreatments peptides are represented in bold. N= number of animals. Values are means ± SD.*

Pretreatment	**PBS**	**CGRP8-37**	**CGRP**
CLR-tg (n=2)	89.1 ± 26.6	136.6 ± 1.9	
Wt-(CLR-tg) (n=3)	168 ± 61.1		129.6 ± 86.6
αCGRP-/- (n=3)	173.7 ± 47.7	187.3 ± 41.0	123.9 ± 81.1
Wt (αCGRP-/-) (n=3)	99.8 ± 48.8		74.3 ± 43.1

3.4. Verification of RAMP1- and CLR- RNA expression on keratinocytes

RAMP1- and CLR-subunit expression in mouse keratinocytes was assessed with specific amplification primers and PCR technique. *Fig. 17* shows an agarose gel after gel electrophoresis of amplified cDNA from fetal lungs and keratinocytes of wild-type mouse for the CLR and the RAMP1 subunits of the CGRP receptor. Fetal lungs are used as a positive control, because of their high CLR gene expression (http://commons.wikimedia.org/wiki/File:PBB_GE_CALCRL_206331_at_tn.png)
Specific bands for RAMP1 (249 bp) and CLR (262 bp) (Kroeger et al., 2009) can be observed in mouse keratinocytes, which implicates that cutaneous cells express RAMP1- and CLR-RNA.

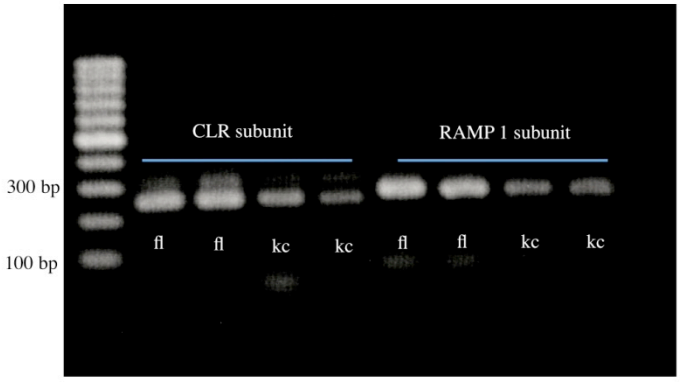


***Figure 17**: Agarose gel of CLR- and RAMP1-subunit. fl: fetal lungs (positive control); kc: keratinocytes*

3.5. Staining and de-staining of mouse skin with ET-1

In order to study the CGRP effect on RhET-1 stained skin, sections of mouse paw skin were stained with RhET-1 and then incubated with CGRP. The fluorescence density changes were calculated. The reason for pre-incubation with BQ788 was to limit the binding of RhET-1 to the ET-A receptor and therefore avoid the involvement of the ET-B receptor, which would interfere with the fluorescence measurements.

Fig. 18 represents a histological section of the skin after RhET-1 staining. The anatomical division of the skin in epidermis (1), dermis (2), and epidermis (3) is clearly identifiable. The layer of interest, where the fluorescence intensity was measured, was the dermis (2). The epidermis (1) is principally composed by dead keratinocytes. The high fluorescence intensity of this layer is a consequence of unspecific binding and for this reason excluded from the measurements.

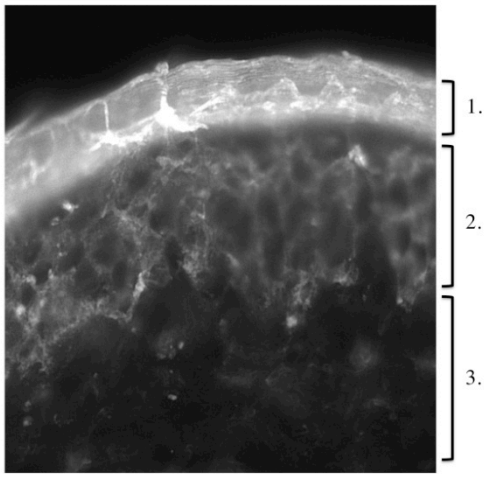
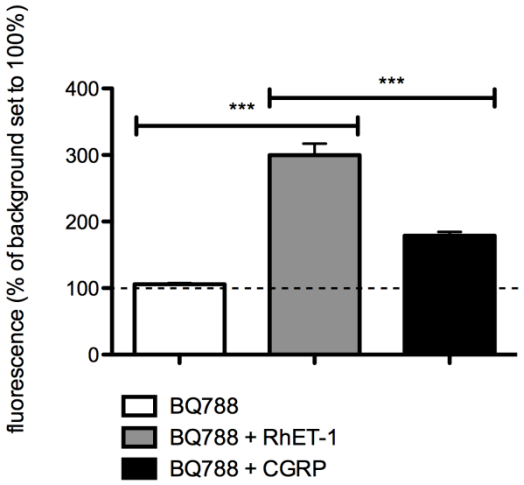


***Figure 18**: Representative image of a histological section of the skin after staining with RhET-1. 1:epidermis; 2: dermis; 3: subcutis*

The background fluorescence was set to 100%, which corresponds to the subcutis fluorescence. Incubation with BQ788 did not cause any changes in the fluorescence intensity, whereas after RhET-1 incubation a 3-fold increase of the fluorescence intensity was observed. The binding of RhET-1 to the ET-A receptor can be partially terminated by CGRP. In fact, the fluorescence intensity decreased by 40%. However, the fluorescence values did not return to the initial baseline values before RhET-1 incubation (*see Fig. 19, A*).

In addition, to test the vulnerability of the RhET-1 staining in time and after washing steps, the experiment was repeated changing the last step (*see Fig. 19, B*). Instead of CGRP, the sections were incubated again with BQ788 and washed with HEPES buffer. No time or washing dependent decrease of the RhET-1 induced fluorescence could be noticed.

A)



B)

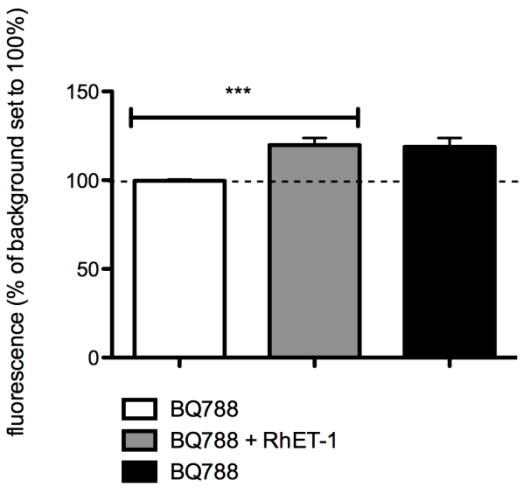


Figure 19*: Fluorescence intensity changes after incubation in sequence with A) BQ788; BQ788+RhET-1; BQ788+CGRP and B) BQ788; BQ788+RhET-1; BQ788. Values are means ± SEM. The symbols denote significant difference between the fluorescence intensities;* $^{***}p<0.0001$ *(n=3, n corresponds to the number of section analyzed per incubation)*

4. DISCUSSION

The aim of the present study was to elucidate if the anti-endothelinergic action of the neuropeptide CGRP described previously for arterial smooth muscle cells (Meens et al., 2009) might also act on nociceptors and keratinocytes, thus representing a peripheral analgetic mechanism of CGRP.

Two animal models (CLR-tg and αCGRP$^{-/-}$ mice) were used to study the role of CGRP during painful stimulations. CLR-tg mice were expected to have reduced pain perception compared to their control. Indeed this was confirmed in all the in-vivo experiments. Compared to wild-type controls, the sympathetic reaction following thermal stimulation during etomidate anaesthesia was reduced and the threshold for mechanical and thermal sensitivity was higher. The induction of neuropathic pain through the constriction of the sciatic nerve did not reveal any difference between the transgenes and their control littermates. However, the time spent with licking and biting the paw after intraplantar ET-1 injection was also decreased in CLR-tg mice compared to the control group. In addition, the shorter licking / biting times in CLR-tg mice were increased after pretreatment with CGRP8-37 and in wild-type mice licking / biting times were reduced after pretreatment with CGRP.

The αCGRP$^{-/-}$ genotype was expected to have an increased nociception. Unfortunately this hypothesis was only partially supported. On one hand, the sympathetic reaction after thermal stimulation during etomidate anaesthesia was increased. On the other hand baseline values as well those obtained after CCI regarding mechanical and thermal sensitivity did not differ from the control mice. In contrast, the intraplantar injection of ET-1 evoked a longer time of licking and biting the injected paw compared to the control mice. In line with our hypothesis αCGRP$^{-/-}$ mice and also their wild-type controls showed reduced licking / biting behaviour after pretreatment with CGRP.

Two additional in-vitro studies confirmed on one side the capacity of keratinocytes to produce CLR and RAMP1 specific RNA and on the other side the ability of CGRP to terminate the complex of ET-1/ET-A receptor in skin sections.

4.1. Thermal stimulation in etomidate anaesthesia and c-fos expression in the spinal cord

Painful stimuli increase the sympathetic tone, which can be objectively measured by an increase of heart rate, blood pressure, skin conductance, corticosteroid concentration, and catecholamine and endorphin concentration (Lamont et al., 2000). The goal of this experiment was to measure the sympathetic response during thermal stimulation, by monitoring of blood pressure and heart rate changes, and to correlate them with the genotype. The $\alpha CGRP^{-/-}$ mice were expected to have an increased sympathetic reaction, which reflects an increased nociception. Indeed, the increase in the mean systolic blood pressure was more than 50% higher compared to control mice. Also the heart rate increase was (nearly three-times) greater as in the control mice. The decreased nociception of the CLR-tg mice has also been confirmed in this experiment. In fact, the increase of the mean systolic blood pressure and heart rate was less than half the increase observed in the control mice. These findings allowed the conclusion that the genotypes $\alpha CGRP^{-/-}$ and CLR-tg influence the sympathetic reaction following thermal painful stimulus i.e. if the neuropeptide αCGRP is missing, the sympathetic response to a nociceptive stimulus is increased whereas an overexpression of CGRP receptors decreases it. Because ET-1 is released from keratinocytes after nociceptive thermal stimuli or mechanical damage, these results support our hypothesis (cf. *Fig. 4*) that CGRP inhibits the ET-1 mediated pain axis through destabilizing the ET-1/ET-A complex (Khodorova et al., 2009b).

Regarding the induction of the immediate-early gene c-fos, no difference were seen between the knockout mice and their control group, while in the CLR-tg mice a lower amount of c-fos expression could be observed compared to the control group. However, the interpretation of c-fos expression and its association with nociception has to be done very carefully. There are some contradictory studies related to the induction of c-fos and correlation to the nociception. A critical review about c-fos studies and the use of this parameter to quantify the nociception process reveals some doubts about its utility and forewarns to directly associate pain and c-fos level (Harris, 1998). C-fos is used as a general marker of neuronal activity and nociceptive processing and is used as a tool for the study of analgetic drugs. But some studies suggest a dissociation of pain from the c-fos expression level in the spinal cord. For

example in rats, cocaine decreases the formalin-induced nociceptive behaviour but not the c-fos level in the spinal cord (Hämäläinen et al., 1996). Another study describes the intraplantar administration of different formalin concentrations, which provoke proportional level of c-fos in the spinal cord (lower concentration of formalin induces lower amount of c-fos positive cells), but the same intensity of pain behaviour, no matter which concentration of formalin is injected (Harris et al., 1995). In addition, the cellular function of c-fos remains not completely understood because the hypothesis that it contributes to the sensitization of spinal nociceptive neurons is due to the fact that c-fos expression is increased after nociceptive stimulation. But a study involving the administration of c-fos antisense oligodeoxynucleotide speculates about an inhibitory function on the pain axis of the c-fos protein (Hunter et al., 1995). Hunter et al. suggest that the pretreatment with antisense c-fos-mRNA, preventing the c-fos protein production induced by formalin, increases the formalin-induced licking-biting behaviour and decreases the production of the dynorphin opioid peptides, which exert an antinociceptive action. In conclusion, c-fos is a marker for neuronal activation but its function is not yet clear. Whether the activation of neurons in the dorsal horn of the spinal cord leads to inhibition or maintaining of nociceptive processes remains unknown. For these reasons the association of c-fos expression with pain has to be done carefully.

Only the CLR-tg mice and their controls were subjects of CGRP8-37 pretreatments. The intravenous administration of 20 µmol/kg CGRP8-37 induces a significant reduction of the sympathetic response following thermal stimulation in the control mice. In wild-types, c-fos level and mean systolic blood pressure and heart rate difference were all reduced by more than half compared to untreated mice.

To best of our knowledge no publication exists describing the pain sensitivity after intravenous injection of CGRP8-37. The studies we found only used intrathecal administration and show consistently its analgesic function (Yu et al., 1996a; Yu et al., 1996b; Salmon et al., 2001; Yu et al., 1994). Because we administered the antagonist intravenously in an about 40-times higher concentration than described in previous studies, we suppose that CGRP8-37 may cross the blood brain barrier and, thus, also act directly on spinal cord neurons. Therefore, the analgesic action of CGRP8-37 in the spinal cord could cover its nociceptive action at the peripheral

endings of the nociceptors. No study describes the permeability of CGRP or CGRP8-37 at the blood brain membrane, however we suppose that its permeability could be similar to that of amylin, because both CGRP and amylin are composed by 37 amino-acids and share around 50% sequence homology (Brain & Cambridge, 1996). The accumulation of amylin in the brain is about 0.12% of the injected doses per gram brain tissue (Banks & Kastin, 1998). To assess the amount of CGRP8-37 crossing the blood brain membrane in our experiment we have to estimate the amount of applied CGRP8-37 in relation to the CGRP plasma concentration. Also here, no literature is known about the CGRP plasma concentration in mice. Considering that in men plasma concentration of CGRP is 50 pmol/L (Birklein et al., 2001; Hasbak et al., 2002; Schifter et al., 1995; Born et al., 1991), in mice the total amount of CGRP (for a plasma volume of about 0.8 ml (Vogel et al., 2003)) is about 0.04 pmol and in rats 0.35 pmol. In a study of Khodorova et al. the injected CGRP8-37 dose was 18 nmol (Khodorova et al., 2009a), which would correspond to 51'000 times the normal total amount of CGRP present in the plasma. If the hypothesis that the permeability of CGRP8-37 through the blood brain barrier is similar to amylin, namely 0.12% of the injected dose per gram brain tissue (Banks & Kastin, 1998), the injection of 18 nmol CGRP8-37 would result in an accumulation of 21.6 pmol/g brain tissue, thus exceeding the CGRP plasma concentration 432 times. These considerations fit quite well to our hypothesis. In fact, the administration of 20 µmol/kg CGRP8-37 resulted in a decrease of the sympathetic response after painful stimulation, whereas the subcutaneous administration of only 0.2 nmol CGRP8-37 resulted in a pro-algesic action of CGRP8-37 (*see "3.3: Licking biting time after intraplantar ET-1 injection"*).

CLR-tg mice did not show any changes in mean systolic blood pressure, heart rate or c-fos levels if pretreated with the CGRP receptor antagonist. Considering that the overexpression of CGRP receptors in the periphery causes a decreased activation of nociceptors (because of the termination of the ET-1 mediated pain perception), a lower activation of CGRP receptors centrally may take place as well, which could explain that no effect can be see when CLR-tg mice are pre-treated with CGRP8-37.

4.2. Assessment of the mechanical and thermal sensitivity before and after chronic constriction of the sciatic nerve

The aims of this experiment were i) to assess the baseline values for mechanical and thermal sensitivity of the CLR-tg and the αCGRP$^{-/-}$ mice, ii) to check if the induction of neuropathic pain has any effects on the pain sensitivity compared to the basal values, and iii) if the genotype influences the degree of hyperalgesia provoked by the CCI.

The CLR-tg mice confirm their decreased nociception in both mechanical and thermal baseline sensitivity values. The thermal as well as the mechanical sensitivity of the CLR-tg mice were significantly lower compared with wild-type littermates. These results implicate a genotype effect on the nociceptive threshold in the CLR-tg mice, supporting our hypothesis. In contrast, the αCGRP$^{-/-}$ genotype did not differ from its control group in the baseline values of mechanical and thermal sensitivity. The hypothesis that the lack of αCGRP could induce an increased nociception, because interaction with the ET-1-mediated pain axis is not possible was not supported in this case. However αCGRP is not only missing in peripheral endings of the nociceptors but everywhere in the body and important here also in the central endings of the nociceptors. It is known that CGRP has a pro-algesic action in the spinal cord, thus, the missing of the pro-algesic action of CGRP in the spinal cord could compensate for the hypothesized analgesic action of the same peptide in the periphery. In this regard, our results are in accordance with a previous study which showed that basal values of thermal pain sensitivity of the CT/αCGRP$^{-/-}$ mice (here both calcitonin and CGRP are knocked out) also did not show any difference compared to the CGRP$^{+/+}$ genotype (Zhang et al., 2001).

The degree of hyperalgesia developed after CCI was expected to be lower in CLR-tg mice and higher in the αCGRP$^{-/-}$ mice, but both genotypes did not differ in the degree of developed hyperalgesia compared to their related control. Surprisingly, the decreased nociception demonstrated by the CLR-tg in the baseline values mice was suppressed after CCI, too. The ligatures cause the swelling of the nerve and a primary and secondary hyperalgesia may take place (Meyer et al., 2006). Primary hyperalgesia is located on the site of injury and is mediated by the sensitization of primary afferent nociceptors, while the secondary hyperalgesia occurs in the uninjured tissue and is

thought to be due to sensitization in the central nervous system (Campbell & Meyer, 2006). The consequent condition of neuropathic pain and neurogenic inflammation influences in turn the level of CGRP in the spinal cord. A study reports that CGRP levels decrease 31 days after CCI (Kajander & Xu, 1995), whereas another one shows that the CGRP-immunostaining levels do not change during the development of neuropathic pain (Garrison et al., 1993). These studies show that changes in the CGRP content in neuropathic models are controversial and this can partly be explained by the constriction technique. In fact, one critical point of realizing the sciatic constriction as described in Bennet and Xie (Bennett & Xie, 1988) is that the inter-animal variability is quite high because of the different tightness of the ligation. Mosconi and Kruger compared the CCI method of Bennet and Xie with the utilization of a fixed-diameter polyethylene cuff, concluding that this latter seems to be more consistent in the magnitude of nerve injury assessed by the alteration of fiber spectrum (Mosconi & Kruger, 1996). In addition, also the material used for the constriction seems to play an important role (Xu et al., 1996). As a consequence, standardization of the CCI appears to be an important issue to obtain coherent results within a group and more objective values in future experiments. It is therefore possible that a genotype effect is masked by the too big inter-animal variance introduced by the CCI technique used in the present study. In addition, we postulate that CGRP should have an analgesic effect in the periphery. However, centrally released CGRP has the opposite function as it is known that CGRP8-37 is able to reduce mechanical and thermal hyperalgesia in chronic central neuropathic pain provoked by spinal hemisection in a dose-dependent manner (Bennett et al., 2000). Thus, an analgesic function of CGRP in the periphery as we suggest may be covered by centrally and simultaneously released CGRP that contributes, together with SP, to the central mechanism of hyperalgesia (Galeazza et al., 1995).

Finally, we used the contralateral paw as a control of the ipsilateral paw after CCI. Perhaps a sham surgery, namely the exposing of the right sciatic nerve without ligating it, would be a better control than just the unharmed contralateral paw, because a certain degree of soft tissue trauma can also influence the difference between the injured (nerve constricted) and the uninjured paw.

4.3. Licking biting time after intraplantar endothelin-1 injection

In rodents, intraplantar injection of endothelin-1 (ET-1) induces mechanical allodynia (Baamonde et al., 2004; Balonov et al., 2006; da Cunha et al., 2004). Because in our hypothesis the CGRP released from the nociceptors inhibits the binding of ET-1- to the ET-A receptor and therefore stops the ET-1 mediated sensation of pain (*see introduction*), this experiment was essential to demonstrate the interaction between the different peptides and receptors.

The goal of this experiment was to assess the nocifensive behaviour after intraplantar injection of ET-1. The time spent in licking or biting the injected paw was observed for 75 minutes after ET-1 administration. As these experiments are preliminary no statistical analysis was performed.

The intraplantar injection of ET-1 results in an increase of the sodium concentration in the nociceptors, which leads to depolarization and consequently to pain perception (Khodorova et al., 2002). Because we think that the central actions of CGRP and its antagonist are opposite as in the periphery, we employed a 100-fold lower amount of CGRP and CGRP8-37 (0.2 nmol) as described previously in the literature (Khodorova et al., 2009b) to avoid or at least minimize the peptides to cross the blood brain (or spinal cord) barrier. In addition, the peptides were injected subcutaneously under the back skin to prevent a high concentration peak in the plasma and to increase the effect duration of the peptides due to the slower release from the subcutaneous depot.

Comparing the licking / biting time after ET-1 injection we could observe a genotype effect in both CLR-tg and $\alpha CGRP^{-/-}$ mice when pretreated with PBS. CLR-tg mice were involved in licking and biting the injected paw nearly half the time compared to the wild-type littermates. On the other hand, the $\alpha CGRP^{-/-}$ mice spend about 75% more time in licking/biting the ET-1 injected paw compared to their wild-type littermates. These preliminary results show the tendency of an increased ET-1-induced nociception by the αCGRP deficient mice and a decreased one in the CLR-tg mice. In line with the genotype effect we observed that CLR-tg mice pretreated with CGRP8-37 showed an increased licking / biting behaviour (53%) compared to the PBS pretreated CLR-tg mice. In contrast, pretreatment of $\alpha CGRP^{-/-}$ mice with the antagonist showed nearly unchanged (+7.8%) licking / biting times. This is explained by the fact that $\alpha CGRP^{-/-}$ mice do not express the neuropeptide αCGRP, and so it seems logic that antagonizing a peptide that is lacking has no effect. At last,

pretreatment with CGRP resulted in a mean decrease of 26% of the licking and biting time of both wild-type group as well as the αCGRP$^{-/-}$ mice when compared to PBS pretreated animals.

The importance of this experiment is given by the fact that it is the most direct behavioural test of our hypothesis because here the direct effect of ET-1 and its interaction with CGRP is studied *in vivo* and in the tissue of interest. These preliminary results are very promising and nicely support our hypothesis of a peripheral analgesic action of CGRP.

4.4. Verification of RAMP1- and CLR- RNA expression on keratinocytes

The goal of this experiment was to assess the CGRP receptor expression in keratinocytes. Indeed we found the specific mRNA for CLR- and RAMP1-subunits in keratinocytes using RT-PCR and agarose-gel-electropheris as evident from the specific bands for RAMP1- (249 bp) and CLR-subunit (262 bp) (Kroeger et al., 2009). Although the Central Dogma of biology "DNA makes RNA makes protein" implicates that there is a correlation between RNA and protein level, this correlation coefficient of RNA and protein can vary strongly and it depends, among others, from the cell-type and the gene of interest (Guo et al., 2008; Gry et al., 2009). Indeed, the specific bands for CLR and RAMP-1 are an indication that the CGRP receptor RNA is expressed in keratinocytes, but the question if this RNA is translated in protein remains unanswered. The verification of CGRP receptor on a protein level implicates good and specific antibodies for both receptor components, which unfortunately are not commercially available so far.

Alternatively, we tried to detect CGRP receptors with radioactively labelled CGRP, performing a binding study with freshly isolated keratinocytes (data not shown). Very low or no binding at all was observed. This however does not ultimately mean that the CGRP receptor is not present because the trypsin used during the isolation of keratinocytes to separate the dermis from the epidermis might have damaged the surface receptors, hampering the binding of radioactively labelled CGRP.

4.5. Staining and de-staining of mouse skin with ET-1

In this experiment we investigated the interaction between ET-1, CGRP, and their respective receptors. The purpose was to assess if CGRP is able to terminate the binding between ET-1 and ET-A receptor in the cutaneous cells, as described for mesenterial arteries by Meens et al. (Meens et al., 2009). After incubation with RhET-1 we observed a 3-fold increase of fluorescence compared to the values after incubation with BQ788, an ET-B receptor antagonist. This increase of fluorescence is partially reduced after incubation with CGRP (to 40%) and may be the result of an interaction between activated CGRP receptors and ET-A receptors. Thus, this experiment suggests that functional CGRP receptors are present on keratinocytes.

The fluorescence intensity after CGRP incubation did not return to the initial value, which means that not all the RhET-1 could be removed from the ET-A receptor. One explanation of the partial effect of CGRP could be the fact that the skin was frozen to allow cutting and then thawed during the experiment, which could have damaged the complexes of the different receptors and/or their components including their coupling to the second messengers such as G-proteins on the plasma membrane, impeding their functional interaction. Also in the control experiment performed with fresh arteries the fluorescence level after CGRP incubation did not return to the initial level. Whether this is also due to tissue damage that might have occurred during the isolation of the arteries and handling during the experiment is difficult to say. On the other hand, although we used BQ788 to prevent RhET-1 from binding to the ET-B receptor this blocking might not be 100%. Moreover all peptides have some unspecific binding that cannot be avoided. The experiment should be repeated with a greater number of sections to confirm the mechanism described. The comparison between wild-type skin and skin of CLR-tg or $\alpha CGRP^{-/-}$ mice would also be interesting.

Alternatively to employing histological sections one could use cultured keratinocytes and analyse them with flow cytometry, which is more sensitive, because any single cell is analysed based on its fluorescence. However also for such experiments the problem of cell separation remains and consequently an alternative method to separate the cells from culture dishes or from skin samples should be considered to bypass the

use of trypsin, which, as mentioned in *"4.4. Verification of RAMP1- and CLR- RNA expression on keratinocytes"*, might damage the cell surface receptors.

4.6. Overall conclusion and outlook

In this study we have investigated the role of CGRP in the ET-1 mediated pain axis.

The focus of the experiments was the characterization of the nocifensive behaviours of the CLR-tg and the αCGRP$^{-/-}$ mice during painful stimulation, concentrating on a possible genotype effect.

We partly demonstrated that CGRP has an analgetic effect in the periphery, but not all performed experiments revealed a difference between the genotypes and their control littermates. Because of the various functions of CGRP in the body and its neuromodulatory role in processing nociceptive signals at different levels of the central processing of pain sensations, it is quite difficult to dissect the detailed role of CGRP in the peripheral nociceptive transduction process.

The present study provides some hints for an additional function of CGRP in the process of pain sensation that might be a starting point for future studies regarding the peripheral analgesic action of CGRP. This requires alternative techniques and additional experiments and also other mouse models to better characterize this hypothesis. Regarding additive studies, we were investigating the thermal and the mechanical sensitivity of our animal models, but we did not perform any experiments evoking pain through chemical stimulation or inflammation. The characterization of pain phenotype is in general only complete if all pain modalities have been tested. Nociceptive behavioural studies after chemical stimulation (i.e. intraplantar injection with formalin) or after induction of peripheral inflammation were beyond the time frame of the present study and therefore missing in the characterization of the nocifensive behaviours of the CLR-tg and the αCGRP$^{-/-}$ mice.

Future experiments should also include tissue specifically genetically modified mice. Nowadays, by using the Cre/loxP system the gene of interest can be knocked out in specific cell populations, depending on the promotor used. In our case, the conditional deletion of the CGRP receptor could be performed in the spinal cord neurons and glia

cells (with HoxB8-Cre) and in nociceptors (SNS(Na_v1.8)-Cre), this way avoiding side effects evoked by a classic knock out of a gene in the whole body. Most likely the use of this animal model could help to better characterize the function of CGRP and its receptor during pain perception.

In conclusion, specific antibodies for the CGRP receptor components would be a very precious tool for the description of the anatomical distribution of the CGRP receptor on keratinocytes and peripheral endings of nociceptors and its co-localization with the ET-A receptor. As companies continuously develop new antibodies there is hope that such tools might be available in near future. Alternatively, however, the characterization of the CGRP binding sites after different pain stimulation would be of interest.

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Appendix 1

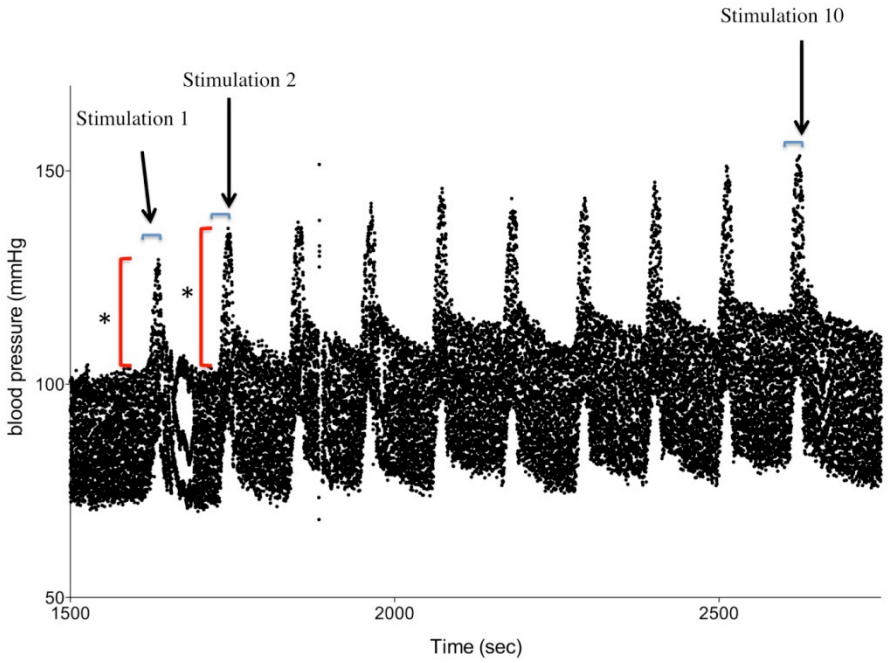

***Figure 20**: Example of a blood pressure curve during thermal stimulation in etomidate anesthesia. In blue: stimulation time of 10 seconds (10 stimulations in total). In red: increase of blood pressure during stimulation. *difference of the systolic blood pressure, which correspond to the highest systolic blood pressure during stimulation minus the systolic blood pressure immediately before the stimulation. For the statistical analysis the mean of the difference of the systolic blood pressure during the 10 stimulations was used.*